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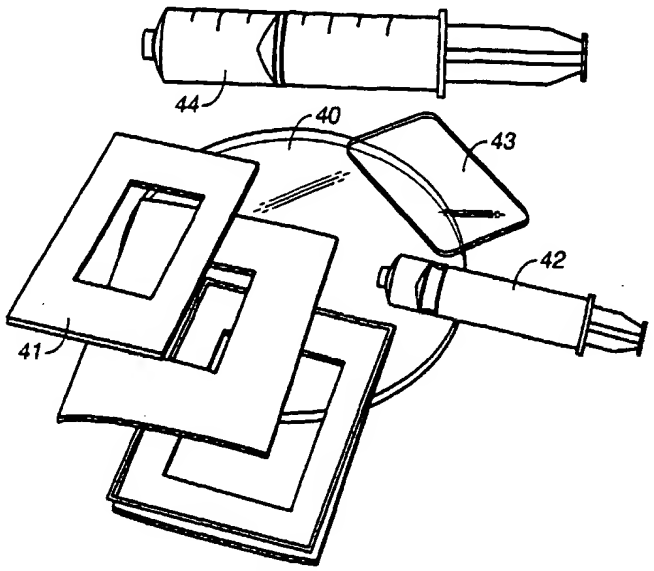
(54) Title: FABRICATION OF THIN SHEET BIO-ARTIFICIAL ORGANS



(57) Abstract: In one embodiment of the present invention, a method is provided for making a physiologically active and biocompatible cellular implant for implantation into a host body. The method includes the steps of: (a) forming first and second layers of first and second polymer solutions, respectively, each layer having a first substantially uncross-linked surface and an opposing second cross-linked surface; (b) forming a sandwich of a cell suspension layer of physiologically active cells in a substantially uncross-linked third solution between the first and second, and (c) cross-linking the first and second polymer solutions in a direction toward the cell suspension layer, thereby forming a cellular implant. In another embodiment, all polymer solutions initially are uncross-linked and sequentially spread in layers followed by cross-linking.

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 855 613 A (DORIAN RANDEL ET AL) 5 January 1999 (1999-01-05) cited in the application examples 4-6	1-30
A	US 5 976 780 A (SHAH KUMARPAL A) 2 November 1999 (1999-11-02) column 2, line 11 - line 59 example 2 figure 1	1-30

☐ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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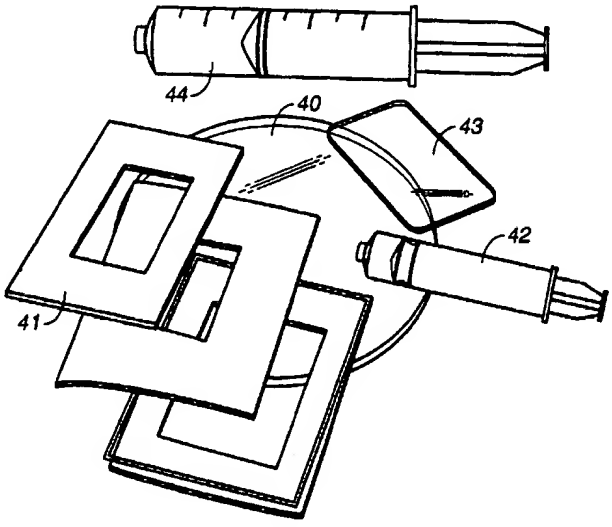
(54) Title: IMPROVED METHODS FOR FABRICATION OF THIN SHEET BIO-ARTIFICIAL ORGANS



(57) Abstract: In one embodiment of the present invention, a method is provided for making a physiologically active and biocompatible cellular implant for implantation into a host body. The method includes the steps of: (a) forming first and second layers of first and second polymer solutions, respectively, each layer having a first substantially uncross-linked surface and an opposing second cross-linked surface; (b) forming a sandwich of a cell suspension layer of physiologically active cells in a substantially uncross-linked third solution between the first and second, and (c) cross-linking the first and second polymer solutions in a direction toward the cell suspension layer, thereby forming a cellular implant. In another embodiment, all polymer solutions initially are uncross-linked and sequentially spread in layers followed by cross-linking.

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IMPROVED METHODS FOR FABRICATION OF THIN SHEET BIO-ARTIFICIAL ORGANS

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention concerns methods for making thin sheet bio-artificial organs for use in treatment of disease. Thin sheet bio-artificial organs are devices for surgical implantation which entrap cells or tissue producing desirable substances or having desirable properties. Specifically, the field relates to thin sheets containing cells and which have dimensions and physicochemical properties allowing maintenance of tissue viability through rapid diffusion of nutrients and oxygen, and affording protection from contact of said cells with cells of the recipient's immune system, said sheets optionally having the further properties of substantially excluding factors necessary for humoral immune destruction of the entrapped cells and having the additional properties of biocompatibility, mechanical strength and chemical stability sufficient that the entrapped cells or tissue can function in vivo for a long time.

Bio-Artificial Organs

The potential utility of bioartificial organ implants to treat disease has been long recognized. The exemplary bioartificial organ is the bioartificial pancreas containing insulin-producing islets of Langerhans, for which there is great clinical need in the treatment of diabetes. Evidence that

islet transplantation can normalize diabetic blood sugars and arrest and reverse vascular decay continues to mount[1]. In essence, the bioartificial pancreas would make possible the benefits of islet cell transplantation without the need for immunosuppression. However, the failure of any bioartificial pancreas to be commercialized after decades of research emphasizes the difficulty of the task.

The background of this invention was described in a related patent, No. 5,855,613, incorporated herein by reference.

Recent advances in the bioartificial pancreas field have been in making smaller and more biocompatible microcapsules. A microcapsule is so small that each capsule contains a single islet. Macrocapsules such as sheets comprise many islets each. Advances in the art of making sheet bio-artificial organs in the past four years (and macrocapsules generally) are few and have characteristics such that the essentials of the prior art have changed little. Therefore, in the remainder of this background section we describe only developments since the prior application.

Biocompatible Materials

As described in patent No. 5,855,613, the material of choice for manufacture of bioartificial organs is "bioinvisible," that is, the material by itself or in combination with living tissues does not produce foreign body reaction or fibrosis when implanted in a host organism. One known material for this purpose is highly purified alginates. For example, Van Schilfgaarde et al.[2] observe: "Graft failure cannot be readily explained by immunological rejection, since success rates in rats are similar with isogenic and allogenic encapsulated islet transplantation. Insufficient biocompatibility of the capsules is usually considered to be the main cause...." Recently additional methods for purification of alginates have been reported[3], as well as methods for analysis of the

biocompatibility (and "bioinvisibility" as defined in patent No. 5,855,613) of alginates[4].

Coverage of 100% of Cells

Some devices do not cover the entire islet surface. If even a small bit of the islet is uncovered, macrophages can infiltrate and destroy the entire islet. The cellular attack and destruction sensitize the immune system, leading to a humoral (antibody) response, which may then destroy even those cells that are completely covered. Thus, complete coverage of all of the islets is required to protect the islet cells from both cellular and humoral immune responses.

Previous devices have failed to ensure complete coverage of all islets while at the same time meeting the dimensional constraints imposed by oxygen requirement. This problem is increasingly recognized; for example, Webber et al.[5] observe: "We observed that a small percentage (2-5%) of the microcapsules are obviously defective, being misshapen, oblong or fractured, immediately after their preparation. In addition, we have found that islets or islet cells are either attached to or embedded in the microcapsule's membrane.... We have postulated that these microcapsule defects may allow islet exposure and host sensitization to the graft, as well as reducing durability of the microcapsule's membrane."

Nutrient Diffusion and Bioartificial Implant Dimensions

A successful bioartificial implant must have dimensions that permit sufficient diffusive flux of nutrients into the implant and secretion of bioactive agents out of the implant. Yet the vast majority of bioartificial implants described in the literature have dimensions too large to permit sufficient diffusion. The nutrient limiting cell viability and functionality in bioartificial organs is usually oxygen. The oxygen available at the center of the bioartificial organ (where oxygen is at its lowest concentration) is governed by

the density of oxygen consuming tissue in the bioartificial pancreas, the geometry of the device, diffusivity of oxygen through the bioartificial organ and oxygen tension in the surrounding tissues[6].

Further evidence supporting the importance of geometry and surrounding oxygen tension to the survival of islets has been reported. For instance, Benson et al[7]. reported that cells of the mouse insulinoma line βTC3 "proliferate while they are entrapped in . . . alginate beads. During this process, cell aggregates develop in the bead periphery, which increase in number and size with time," supporting the concept that the higher available oxygen at the surface is crucial for cell growth. Papas et al[8]. report "Encapsulated cells may experience hypoxic conditions postimplantation as a result of one or more of the following: the design of the construct; the environment at the implantation site; or the development of fibrosis around the construct.... Results show that, upon decreasing the oxygen concentration in the surrounding medium, the encapsulated cell system reached a new, lower metabolic and secretory state." Davalli et al.[9] report a dramatic decline in beta cell mass following islet implantation into nude mice and say that "anoxia may be a major factor."

Other studies have attempted to improve oxygen available to cells in the bioartificial organ by electrochemically generating oxygen in situ[10], inducing a vascular bed in the adjacent tissue with VEGF[11], and by increasing the diffusivity of oxygen by addition of perfluorocarbon[12].

Cell Density

The importance of high cell density in a practical bioartificial organ is now increasingly recognized. As Suzuki et al[13]. commented:

> "These results strongly suggest that ways must be found to improve the packing density of

> macroencapsulation devices . . . [T]he surface area of the membrane required to support a given mass of islet tissue is so high as to be impractical for surgical implantation."

Delaunay et al[14]. made much the same point:

> "The design of a geometry to accommodate the number of islets necessary to treat diabetes in larger mammals is certainly one of the major challenges to be overcome in the development of a bioartificial pancreas . . . "

Calafiore's group[15] also recognized the seriousness of this problem ("it was, in fact, found that a viable human islet cell quantity . . . would take, upon microencapsulation [in 700 μm microcapsules] a final graft volume of approximately 180 mL, thereby creating quite serious technical implant problems") and worked to make much smaller microcapsules.

Cellular Trophic Factors

Islet cell mass, viability and functionality may be improved and enhanced by adding cells and/or other substances in the bioartificial implant composition. Recent publications include various trophic cells and substances in the core of the bioartificial organ to enhance the viability and functionality of the cells, including Sertoli Cells[16].

Use of Growing Cell Lines

Islet and other primary cells are characterized by very slow rates of cell division, usually limited to replacement of cells that die naturally. Embodiments of the thin sheets described herein can optionally be made that comprise rapidly dividing cell lines entrapped in rigid capsules, for example alginate-poly-lysine. This can be done, for example, by the method of Cochrum, Dorian and Jemtrud[17].

Previous Approaches

Numerous bioartificial implants have been described; most of these were discussed in patent, No. 5,855,613. The following discussion is limited to passive diffusion type implants each containing multiple islets, and does not include individually encapsulated islets or vascular devices.

Lanza and Chick[18] summarized the state of the art:

> Despite encouraging results [with diffusion-based chambers], a number technical and safety issues...must be addressed....includ[ing] long-term biocompatibility, membrane breakage, and suitability for retrieval.

Several publications described further research on devices discussed in patent No. 5,855,613, including work by Geller[19], Ohgawara[20], Suzuki[13], Tatarkiewicz[21] and Trivedi[22].

The Ohgawara paper greatly expanded the information available on the device compared with the earlier paper cited in patent No. 5,855,613. The planar chamber is 40 mm diameter and 5 mm thick, fabricated from two membranes made by Nucleopore Corp. The tissue density reported was $8X10^6$ cells/1.5 mL. Although the paper claims that there is no fibrosis, the relevant micrograph (Fig. 7) shows a fibrotic mass about 40-50 μm thick. Suzuki et al[23]. reported on the Baxter double-membrane design, but in greater detail than ever before. The Suzuki paper described a flat device substantially similar to the Scharp device (from the same supplier, Baxter) discussed in the prior patent.

It should be noted that many of these devices fail because the polymeric membranes employed significantly reduce oxygen diffusivity and are not amenable to supporting high densities of entrapped cells even when thickness is minimal.

Since the review in patent No. 5,855,613 only four new sheets or planar diffusion systems have published, from Baetge, Dionne, Tatarkiewicz and Usala.

Baetge et al.[24] described a flat sheet sealed double-membrane with loading port. Each membrane was 100 μm thick with a 200 μm core, and thus a total sandwich of 400 μm thickness. The disc was 10mm diameter. Thus, with 3.5 X 10^6 cells the packing efficiency in the core is 30%, or 15% in the total sheet.

Dionne[25] describes imunoisolatory vehicles with a core and permselective jacket, including flat sheets. The methods described may be distinguished from the present invention in that the flat sheet fabrication method of Dionne does not work with alginate. In addition, in the method of Dionne the membranes are not laminated to the core allowing changes in dimensions post implant.

Tatarkiewicz at al.[26] describe a polylysine coated alginate slab, 1mm thick, 0.8 cm^2. They report that only 4000 islets survive when up to 8000 are incorporated. The calculated tissue density is 8%.

Usala[27] describes an implant in the form of a plate with multiple wells, each filled with a cell-collagen matrix, the entire implant coated with poly-para-xylene.

U.S. Patent No. 5,855,613 discloses making the bioartificial implant using a series of molds constructed with frit materials that can be molded or milled, and membranes. This allows diffusion of chelating agents (e.g., sodium citrate) or multivalent cation gelling agents (e.g., calcium, barium or strontium chlorides) to liquify and gel, respectively, the core, coat and overcoat. The manufacturing process makes use only of materials known to be compatible with the implanted cells. The shape of the core, coat and overcoat are molded while the alginate is liquid. The cross-linked core and coats (and later the coat and overcoats) are bonded together by

simply contacting the cross-linked surfaces with a small amount of chelating agent (e.g., sodium citrate). The chelating agent diffuses into the gelled layer and partially liquefies it. The layers are brought into contact with each other. When a cationic cross-linking agent (e.g., calcium, barium or strontium chlorides) is subsequently added, a tight bond is formed between the layers. The outer surface is made very smooth through the simple step of wetting the mold with cross-linking agent solution before contacting the mold with the cross-linked coat or overcoat. This assures that the outer surface is as smooth as the mold surface, limited only by machining of the frit.

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by substantially uniformly spreading said cell suspension over said first layer exposed surface, and

(c) forming a third layer of a third substantially uncross-linked polymer solution on said cell suspension second layer by substantially uniformly spreading said third polymer solution on said cell suspension second layer.

We have invented rapid and convenient methods for fabrication of a bioartificial implant of dimensions never before achieved, with many attendant advantages. For example, objects of the present invention (shared with the previous invention) include a bioartificial implant in a thin sheet configuration that:

- is easily retrievable from the host;
- permits high tissue densities;
- permits diffusion to the tissue cells of the amounts of nutrients, oxygen and other substances required for cellular health, longevity and effective function after implantation;
- comprises viable, physiologically active, tissue cells for implantation in a device which is physiologically acceptable to the host and which effectively provides prolonged protection of the tissue cells, after implantation, from destruction by the host immune system;
- may contain a mesh or support polymer to improve physical properties of the sheet;
- may contain trophic agents such as nurse cells, nutrients, hormones or oxygen carriers to support the cellular health, longevity and effective function of the implant after implantation.

An object of the present invention is to ease scaling up methods for fabrication of larger sheets. A single simple

device may be used to make sheets ranging from very small up to a limit imposed only by the spacing of shims.

Another object of the present invention is to reduce the processing time for making a bioartificial implant. Prior methods relied on many steps to liquify gelled alginate, time for the liquids to diffuse, then addition of chelating agent to gel the alginate again. The new process does not contain any chelation step.

Another object of the present invention is to fabricate the coat and overcoat of a bioartificial implant in a single step. Optionally, an overcoat may be added in an additional step.

Another object of the present invention is incorporation of rapidly dividing cells into thin sheets without risk of the cells bursting out of the sheet.

Another object of the present invention is to control the thickness of the coat/overcoat by physicochemical means, not relying on a mold. This is accomplished by controlling the composition of the first polymer and cross-linking solution so that the layers are substantially cross-linked to a depth of only several microns or by sweeping a straightedge over shims.

The methods of the present application allow for excellent cross-linking between layers, smoother outer surface, easier scale up and more rapid sheet fabrication.

Cells or tissues are entrapped within a core of gel. The core is laminated and firmly bonded to thin coating and overcoating gel layer(s) which do not contain cells or tissue. Sheets of several polymers are possible. For reasons of biocompatibility and convenience of fabrication the hydrogel alginate is preferred.

Detailed Description of the Preferred Embodiments

The utility of this invention is not limited to encapsulation of the islets of Langerhans in a sheet ("islet sheet"). Any cell that secretes a substance with therapeutic value may be used. For example, primary parathyroid cells may be used to treat parathyroid hormone deficiency, or erythropoietin secreting cells may be used to treat anemia or cytokine secreting cells may be used to modulate immunity. Another form of utility would be encapsulation of cells that transform or metabolize substances found in the body. For instance, hepatic cells may detoxify toxic compounds, or cells may be used to oxidize compounds such as ethanol when they are present in undesirable amounts. For all such uses cells may be, for example, primary cells, cultured cells, or genetically engineered cells. Mammalian and non-mammalian cells including prokaryotes may be used.

The dimensions of the bioartificial implant are such that cell viability may be maintained by passive diffusion of nutrients, and preferably, such that a high cell density can be maintained. The dimensions of the bioartificial implant are also such that the bioartificial implant is macroscopic and is easily retrievable from the host and is large enough to contain a significant fraction of the tissue required to achieve the desired therapeutic effect. Such high cell density makes practical surgical use of bioartificial organs possible.

The permeability of the bioartificial implant is such that passive diffusion of secreted cell products permits rapid response to changing physiological conditions. At the same time, the permeability of the membrane sufficiently impedes diffusion of antibody and complement to prevent killing of the implanted cells, even when the tissue is a xenograft or the host is presensitized to the implant tissue.

The bioartificial implant is biocompatible, meaning it produces minimal foreign body reaction. We have found that only

implants that are neutral (causing neither fibrosis nor neovascularization) have been shown to last over a year with minimal decay of function.

In one preferred embodiment, the dimensions of the present bioartificial implant, when in a thin sheet configuration, are such that the surface area of a side of a sheet is at least 30 mm^2, preferably at least 2.5 cm^2 and more preferably at least 10 cm^2, as defined by either (a) the diameter (if the sheet is circular) or (b) the area determined by the method of converging polygons. Although the maximum dimensions can be those which are tolerated by the patient into whom the implant is placed, for ease of fabrication and economy of implanted cells, a suitable surface area of a face of the present thin sheet implant may be 400 cm^2, more preferably 300 cm^2 and most preferably 250 cm^2 (for a human patient with type 1 diabetes). (A smaller sheet would be sufficient for a more potent hormone such as erythropoietin.)

In the bioartificial implant the cell density is that which can be contained within the entire implant. Preferably, the cell density is at least 10%, more preferably 20% and most preferably at least 30% by volume.

The bioartificial implant is sometimes described using the terms "core," "coat" and "overcoat." The core comprises the living tissue, optional trophic factors or nurse cells, alginate polymer cross-linked with a multivalent cation such as calcium, and an optional support polymer such as collagen or fiber mesh for strength. The coat comprises alginate polymer (optionally of different chemical composition) cross-linked with a multivalent cation that partly serves to control permeability. The optional overcoat comprises alginate polymer (optionally of different chemical composition) cross-linked with a multivalent cation that serves to render the bioartificial implant biocompatible. Use of polymers of different chemical composition may require other methods for

cross-linking, such as coval nt bonding or phase change by cooling.

Previous approaches have responded to inherent limitations on diffusion of oxygen by lowering cell densities within the bioartificial implant. We have found that an effective implant must have medium to high tissue densities to minimize the volume of the total bioartificial implant, and that the thickness of a sheet or slab must be very small to permit effective oxygen diffusion. The sum of the core, coat and overcoat thicknesses preferably is less than 500 μm, more preferably 350 μm or less, and most preferably no more than 300 μm. The coat and optional overcoat thickness preferably is minimized so that the tissue quantity may be maximized. We have found that preferable coat and overcoat thicknesses may be from 5 to 100 μm thick, more preferably from 10-80 μm, and most preferably from 10-50 μm. The length and width of the bioartificial implant on the other hand preferably is maximized to permit the greatest possible volume of living tissue to be included in the bioartificial implant and to permit easy retrieval but not so large as to be surgically impractical.

The thin sheet bioartificial organ made according to the present invention typically includes an implant core having a thin sheet configuration comprising viable, physiologically active tissue or cells and a cross-linked alginate gel and optionally, trophic factors and nurse cells, and optionally, a fiber mesh support, being completely covered by an acellular biocompatible coat and optional overcoat of alginates. The alginates are preferably free from fibrogenic concentrations of impurities. The bioartificial implant may have a coat and overcoat to control permeability and enhance biocompatibility. The implant sheet is thin and may be permeable enough to provide a physiologically acceptable oxygen tension at the center of the sheet when implanted in a suitable site in a human or animal subject. The thinness and permeability of the implant allow diffusion of nutrients, especially oxygen, metabolic waste products and secreted tissue products. The

implant preferably inhibits diffusion of antibody and complement.

In accordance with the present invention, improved methods are provided for making thin sheet bioartificial implants without the necessity of a series of molds constructed with frit materials or membranes that allow diffusion of chelating agents to liquify the gelled core, coat and overcoat. The use only of gelling agents and ions known to be compatible with the implanted cells has been retained. However, the use of membranes that allow diffusion of gelling agents to gel the core, coat and overcoat has been made optional, with diffusion of gelling agents to gel the core, coat and optional overcoat now possible from a single solution.

The use of a polymer which can be reversibly gelled is no longer required. This allows selection from a wider range of polymers.

Although many variations of the method exist, the essence of the process is simple. The shapes of the core, coat and overcoat are molded while the polymer solutions are uncross-linked and in a flowable liquid form preferably at a viscosity less than that of a gum but more viscous than water (e.g., from about 100 to 100,000 centipoise). The coat can be formed by spreading as by sliding a straightedge over two shims to form a liquid layer of uniform thickness. The liquid core can be formed by gently suspending cells into a second liquid polymer solution and sweeping this suspension over the first. Preferably the first polymer solution is more viscous than the second. The second layer is formed with thicker shims defining its thickness. In one embodiment, the layered sheet can be formed and the core and coat are simultaneously cross-linked by simply contacting the two liquid polymer solutions formed in the desired dimensions using a system of shims and straightedges, then adding a cross-linking agent to form a tight bond between the layers.

The outer surface can be made smooth during the coat formation step through the simple means of gelling the outer surface of the coat by first wetting a permeable membrane on which it is formed with cross-linking agent solution or by leaving the coat liquid against a smooth impermeable mold until the final cross-linking step. The thickness of the thin sheet is easily controlled through the use of an impermeable mold of one or two surfaces with shim spacers.

An important feature of these new methods is that it is no longer necessary to apply coat and overcoat in separate steps. We have found that a single step produces a coat that completely covers the cells in the core, inhibits diffusion of complement, and has a biocompatible surface free of fibrogenic properties.

Another important feature of these new methods is that the outer surface can be made smoother and thus more biocompatible.

The new methods do not require the manufacture of custom frit molds for each sheet size and geometry.

Due to their simplicity and the small number of steps the new methods form finished sheets rapidly with less trauma to cells and less change in the cellular environment.

Brief Description of Drawings

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Fig. 1 shows the apparatus for the optical glass casting method.

Fig. 2 is a diagram of the optical glass casting method showing initial diffusion of calcium ions into the layered liquid alginate and islets.

Fig. 3 is a diagram of the optical glass casting method showing complete diffusion of calcium ions into the Islet Sheet.

Fig. 4 shows the apparatus of the hydrophilic membrane method for making sheets at the beginning of a cycle.

Fig. 5 shows the apparatus of the hydrophilic membrane method for making sheets as alginate for the coat/overcoat layer is applied.

Fig. 6 shows the apparatus of the hydrophilic membrane method at the addition of the core, cells suspended in alginate.

Fig. 7 shows the apparatus of the hydrophilic membrane method closing.

Fig. 8 shows the apparatus of the hydrophilic membrane method completely closed.

Fig. 9 shows the apparatus of the hydrophilic membrane method as the finished sheet is withdrawn wrapped in a protective membrane, about to be transferred to a bath of cross-linking agent.

Fig. 10 is a diagram of the hydrophilic membrane method; the membrane is prepared by soaking in a solution of calcium gluconate.

Fig. 11 is a diagram of the hydrophilic membrane method; the membrane is covered with a layer of liquid sodium alginate at the instant of application.

Fig. 12 is a diagram of the hydrophilic membrane method; calcium diffuses from the membrane into the liquid alginate, gelling the alginate.

Fig. 13 is a diagram of the hydrophilic membrane method; two membranes layered with partially gelled alginate squeeze a dollop of liquid alginate containing cells or islets.

Fig. 14 is a diagram of the hydrophilic membrane method; the cell/alginate suspension is flattened and the liquid alginate from the membrane layer is displaced by the advancing liquid alginate from the cell dollop (arrows).

Fig. 15 is a diagram of the hydrophilic membrane method; at the completion of the squeezing process the sheet is of uniform thinness and the liquid portion of the membrane alginate has been displaced from view.

Fig. 16 is a diagram of the hydrophilic membrane method; the calcium diffuses in through both membranes and gels all the liquid alginate into a uniform sheet.

Fig. 17 is a photograph of a thin sheet comprising erythrocytes encapsulated in alginate. The U.S. quarter dollar coin gives scale.

Fig. 18 is an electron micrograph of the surface of an islet containing thin sheet. The surface is smooth. The bumps show that the surface of the sheet is convex over the islets.

Fig. 19 is an electron micrograph of the surface of a thin sheet reinforced with a non-woven fabric. The sheet is fractured to reveal the fabric.

Fig. 20 is a micrograph of an Islet Sheet that has been retrieved after 2½ months sutured on to the omentum of a dog. The islets are stained with dithizone.

Fig. 21 is a photograph of an Islet Sheet as it is being sutured on to the omentum of a diabetic dog described in Example 8.

Fig. 22 is a chart showing the blood sugars of a canine allograft described in Example 8.

Fig. 23 is a chart showing intravenous glucose tolerance tests of the canine allograft described in Example 8.

In one embodiment of the present invention, termed "the casting method," a sheet is fabricated as illustrated in Figs. 1-3. Suitable apparatus for this method illustrated in Fig. 1, included a flat plate (40), preferably with the smoothness of optical glass, shims (41), a straightedge for sweeping solutions and suspensions (43), dispensers for solutions and suspensions (42) and for dispensing calcium solutions (44). Figs. 2 and 3 show the beginning and final stages of diffusion of calcium ions into the sheet.

In the first step of the casting method, a first substantially uncross-linked polymer solution is formed by substantially uniformly spreading the polymer solution onto a solid support surface, preferably one that is flat and smooth, e.g., optical quality glass. A suitable polymer solution is a substantially uncross-linked soluble alginate salt (such as sodium alginate or another monovalent cation salt) in a viscosity (e.g., less than about 50,000 centipoise) which permits it to be readily flowable and spreadable in contrast to a hydrogel (which is cross-linked). The polymer solution (42) is spread as a thin layer on a smooth surface, such as optical glass (40). A convenient means of sweeping out this and the subsequent layers described below is by spreading the alginate across the smooth surface with a straightedge (43) while controlling the thickness by means of guide shims (41). These outer layers should be less than 100 μm so that oxygen flux is not unduly impeded. Optionally, a reinforcing fabric or membrane is

placed on top of the layer (e.g., of the type illustrated in U.S. Patent No. 5,855,613).

In the second step of the casting method, a cell suspension of physiologically active cells in a substantially uncross-linked second polymer solution is substantially uniformly spread over the exposed layer of the first polymer solution. Suitably, the suspension of cells (such as islets or other cells) dispersed in alginate solution is spread across the surface of the layer (or layer overlayed with fabric) using a straightedge and thicker spacing shim, typically 100-200 μm thicker than the first shim. In one embodiment, the alginate used for suspension of particulates is of a lower viscosity (e.g., at least about 5% less) than the underlying alginate layer to minimize disturbing the bottom layer while sweeping out the particulate suspension. Optionally, a reinforcing fabric or membrane can be placed on top of the spread particulate suspension.

In the third step of the casting method, another layer of a third substantially uncross-linked polymer solution (e.g., sodium alginate) is spread over the top of the cell-containing layer (or cell-containing layer and reinforcing fabric) with a straightedge (43) using a shim, typically less than 100 μm thicker than the combined thickness of the prior layers. The over-laying alginate solution can optionally be of a lower viscosity than that of the particulate suspension to minimize disturbance of the suspension during sweeping out of the topmost layer.

The cross-linking solution can be spread across the surface with a straightedge (43) guided by thick shims or allowed to pool freely. The entire laminate can be cross-linked by submerging in a solution of cross-linking ions (e.g., a multivalent ion such as calcium) (44) to cross-link the polymer (alginate). Calcium from the overlying liquid quickly diffuses into the layers (Fig. 2) and gels the entire sheet (Fig. 3).

After cross-linking, the implant can be removed from the support surface.

The entire sheet may be strengthened by immersion in a solution of barium chloride. Barium exchanges with calcium in the sheet, and the stronger barium-alginate bonds result in a sheet that is physically stronger.

The cell-containing layer of alginate is preferably constrained to a smaller area than the under- and over-lying layers so that the edges of the device will be devoid of cells.

The reinforcing fabric or membrane is preferably constrained to an area smaller than the under- and over-lying layers and larger than the cell-containing layer so that it may be used as a surgical cuff without any danger of exposing cells in the cell-containing layer during suturing.

As used herein, the term "cells" includes cells or physiologically active tissue. Such cells are washed in isotonic buffer solution lacking cross-linking agent and resuspended in a soluble polymer solution.

Another embodiment of the invention, termed "the partially pre-cross-linked method" is illustrated in Figs. 4-9.

Suitable apparatus to perform this method includes two flat plastic plates (**51**) supporting two smooth flat glass plates, e.g., formed of optical glass (**52**). The plastic plates are hinged (**53**) so that, when the hinge is closed, the flat plates (**52**) are held apart, preferably spaced equidistant across their facing surfaces by spacers or shims (**54**).

In the first step of the partially pre-cross-linked method, two layers of polymer solutions are formed each having one cross-linked surface adjacent a support surface and an uncross-linked opposed surface. This can be performed with a pair of smooth, absorbent membranes (or a single, folded membrane **55**) wetted

with a solution containing cross-linking agent, such as calcium (see Fig. 10). The membranes may be gel films cast from PEG, polyacrylamide, polysulfone, agarose or other polymers. The wet membranes are laid out onto rigid flat surfaces, such as glass optical windows (52). Excess liquid is blotted or displaced from the membrane surfaces.

The flat surfaces are fitted with spacers (shims or rails) (61) which serve as guides to control thickness of a polymer (e.g., alginate) layer to be spread over the surfaces of the membranes. A liquid solution of polymer, such as sodium alginate, is applied from an impermeable depot resting atop one end of the membranes (61) and spread across the membranes using a straightedge knife (62) guided by the shims (61). (In Fig. 5 the depot and the shims are a single rectangular mask 61.)

As the polymer contacts the wet membrane surfaces (see Fig. 11), a smooth skin of cross-linked gel is rapidly formed at the interface by interaction of the polymer with the cross-linking solution. Gradually over the course of seconds to minutes, the cross-linking agent diffuses from the membrane into the polymer solution, interacting with the latter and producing a front of gelation (see Fig. 12). The degree of cross-linking of the polymer layers decreases in a moving or kinetic gradient from the surface contacting the membranes outward, and is dependent on the composition of the cross-linking solution. Parameters which effect the rate of cross-linking include concentration, counterion, viscosity, and temperature. For example, a dilute solution of a weakly dissociable salt of cross-linker in a viscous solution will result in slower diffusion into the liquid polymer.

By appropriate selection of the membrane thickness and porosity, the composition of the polymer solution, composition of the cross-linking solution and the concentration of the cross-linker in the cross-linking solution, conditions can be established whereby within a minute or so, the polymer layers are substantially cross-linked to a depth of only several

microns. For example, using Gelman 0.2 micron cellulose nitrate filter membranes soaked in a solution of 4.63% sucrose, 3.2% calcium gluconate and 10 mM HEPES, pH 7.0, substantially complete cross-linking of a neutral 4% solution of moderate molecular weight, mannuronate-rich sodium alginate in isotonic sucrose extends to about 30 or 40 microns from the membrane surface, regardless of the total depth of the alginate layer. Under these specific conditions, calcium-binding uronate residues are in excess of available calcium, and further progression of the gel front is governed by calcium exchange between gelled and liquid alginate. This is a relatively slow process because the gelled calcium alginate does not diffuse and diffusion of liquid alginate is slow by virtue of its high molecular weight. Thus, there is a comfortable window of time during which the layer exists in this particular state.

This process may be adapted to polymers that gel as a result of a physical process rather than a cross-linking agent, for example, gelation of a liquid that cools to its gel point.

The next step in the partially pre-cross-linked method is to form a sandwich of an uncross-linked cell suspension layer (e.g., of the type previously described) sandwiched between the exposed uncross-linked surfaces of the two partially pre-cross-linked polymer layers. In one embodiment, the suspension is deposited onto one of the two partially cross-linked overcoating polymer layers (by syringe **71**). The other partially cross-linked layer is lowered on top of the suspension (**81**), contacting it, and is pressed down until the spacer shims which control the distance between the two membrane surfaces (Fig. 8) collide. As the cell suspension is flattened between the opposing layers, the soluble polymer component of said layers is displaced radially (**91**); see Figs. 13 through 15. The gelled and highly viscous, partially gelled polymer components of the layers are unperturbed because of their cross-linked, macromolecular structure, so that the suspended cells are substantially uniformly separated from the membranes by a layer of gelled polymer, the thickness of which

is determined by the extent of cross-linking prior to collision of the shims.

The sandwich consisting of membrane-polymer layer-cell suspension-polymer layer-membrane may then be slid out from between the two rigid, flat surfaces (Fig. 9). This operation can be facilitated by infusing cross-linking solution between the flat surfaces around the sandwich, for instance by using a syringe or suitable device for applying the solution to the space between the surfaces. The presence of a solution around the membrane sandwich both continues the cross-linking process and lowers the surface tension between the damp membranes and the flat surfaces.

The sandwich may then submersed in cross-linking solution (92) and incubated for sufficient time to ensure that polymer is cross-linked entirely throughout its thickness. Alternatively the cross-linking agent can be infused solely between the flat surfaces, or through semipermeable flat, rigid surfaces such as glass frits. The membranes are removed from the cell-containing multilayered polymer sheet and the sheet is equilibrated in an appropriate buffered salt solution or nutrient medium, for example, HEPES buffered normal saline with 5 mM $CaCl_2$, to prepare it for tissue culture or surgical implantation.

The entire sheet may be strengthened by immersion in a solution of, e.g., barium chloride. Barium exchanges with calcium in the sheet, and the stronger barium-alginate bonds result in a sheet that is physically stronger.

Figs. 10-16 illustrate formation of a sheet by the partially pre-cross-linked method (the hydrophilic membrane method). Fig. 10 shows a porous membrane (101) prepared by soaking in a solution of a multivalent cation salt cross-linking agent (e.g., calcium gluconate). Fig. 11 shows the membrane (101) covered with a layer of liquid sodium alginate (102) at the instant of application before any calcium has diffused out of

the membrane. Fig. 12 shows calcium diffusing from the membrane (101) into the liquid alginate (102), gelling the alginate; gelling is complete near the membrane. The amount of calcium in the membrane is chosen so that the alginate remains liquid at a distance from the membrane. Fig. 13 shows two membranes (101) layered with partially gelled alginate (102) beginning to squeeze a dollop of liquid alginate containing cells or islets (103). Fig. 14 shows the squeezing continues as the cell/alginate suspension is flattened (103) and the liquid alginate from the membrane layer is displaced by the advancing liquid alginate from the cell dollop (arrows). Fig. 15 shows the completion of the squeezing process. The sheet is of uniform thinness and the liquid portion of the membrane alginate has been displaced from view. The spacing of the membrane is determined by shims (54). Fig. 16 shows calcium diffusing in through both membranes and gelling all the liquid alginate into a sheet.

Proliferating cells entrapped in a sheet of polymer may break out of the sheet during expansion. In order to prevent this eventuality, such cells may be first entrapped within shells which have sufficient mechanical strength to contain them.

For example, cells which either are free or entrapped within an intraluminal gel matrix can be contained within shells in the form of small, semipermeable hollow fibers, sealed at either end. See, e.g., Sharp et al., *Diabetes* 43(9):1167-70 (1994). These sealed tubes containing cells may be layered onto one of the two partially cross-linked polymer layers of hydrophilic membrane method prior to layering with the apposing layer.

Alternatively, the cells may be entrapped within microcapsules, such as polylysine stabilized alginate microcapsules. In order to ensure complete entrapment within poly-lysine/alginate microcapsules without defects, it is preferable to coat the cells in such a way as to ensure complete coverage of all cells with alginate before stabilization of the alginate with polylysine. This can be done, for example, by the method of

Cochrum, Dorian and Jemtrud (Note 17). Such cell-containing microcapsules, after stabilization by polylysine treatment and optional dissolution of the core gel matrix, can then be substituted for free cells or tissue in the method for sheet manufacture in both the hydrophilic membrane method and the casting method.

The polymer for the cell-containing core may have a different composition from that used in preparation of the partially cross-linked coating layers. For example, to provide for improved mechanical strength without severely limiting permeability, the core can be composed of alginate with a higher guluronate content (such as that isolated from Laminaria hyperborea, F_G=0.68) or a higher molecular weight. The partially cross-linked layers of the hydrophilic membrane method (or the outer liquid layers of the casting method), which will ultimately separate the cell-containing core of the sheet from the external environment, may be composed of a different alginate composition chosen for desired permeability and biocompatibility properties. In general, the characteristics of the polymer solution are described in U.S. Patent No. 5,855,613, except that there is no need for polymers that can be reversibly gelled.

Polymers other than alginate can be used. These polymers must be liquid in one phase (which allows cell viability) and gelled in another phase (which also allows cell viability), for instance by cross-linking by addition of a cross-linking agent (which also allows cell viability). For example, chitosan can be substituted for alginate and cross-linked by similar divalent ion diffusion. Acrylamide monomer may be cross-linked with ammonium persulfate and TEMED (N,N,N′,N′-tetramethylathylenediamine).

Suitable cross-linking agents are multivalent cation salts (e.g., of calcium, barium, strontium, or mixtures thereof). Such salts include as counterions gluconate, lactate, and chloride.

In the case of casting method, an ven greater variety of polymers may be used. For example, a free-radical polymer may be made by sweeping the three layers in the dark (or under red light) then exposing the sheet to light. Similarly, polyacrylamide may be cross-linked with TEMED mixed with ammonium persulfate. Agarose, gelled by a change in temperature can be used.

Essentially any method may be used for cross-linking the polymer solution in the casting method or partially cross-linked method so long as it causes a phase transition from liquid to a gel or solid. In the foregoing description, cross-linking takes place by diffusion of cross-linking agent toward the interface of the cell suspension layer from one or more of the sandwiching layers.

In another form of cross-linking, a temperature-induced phase transition may be used. For example, 1% agarose in physiological solutions undergoes a liquid-to-gel phase transition at approximately 37°C. Cells for encapsulation can be suspended in 1% liquid agarose thermostated at temperatures slightly higher than this, e.g., 40°C. This liquid suspension is used for the core polymer. After formation of a sandwich with substantially cell-free polymer solutions, cooling below 37°C would cross-link and thereby gel the suspension, entrapping the cells, without need for diffusible cross-linking agents.

Alternatively, the substantially uncross-linked polymer solution may be cross-linked by photoactivation using solutions of polymers such as polyethylene glycol derivatized with photolabile cross-linking groups. For example, the chemistry described in U.S. Patent 5,573,934 (Hubbell, et al.) can be implemented in the described methods. In a preferred embodiment, Hubbell describes the use of polyethylene glycol-diacrylates as a soluble "macromer" in which the cells to be entrapped are suspended. To this solution is added ethyl eosin and triethanolamine to act as a photon-induced free radical

donor. Upon illumination with 415nm light the eosin produces a free radical which can be transferred via the triethanolamine to the acrylate group on the macromer, which becomes activated and cross-links with other acrylate groups forming a larger polymer which will no longer be soluble, but will form a cross-linked gel phase entrapping the cells and thereby forming a cellular implant. Other photoactivated cross-linking systems are well-known in the art.

Each polymer will have distinct permeability, stability, and biocompatibility characteristics which can be tailored to specific applications. For long-term cellular implants, we have found alginate possesses the best balance of these properties, especially biocompatibility, but this in no way limits the choices available for this or other applications of this invention.

As briefly described above, to improve mechanical properties of the sheet, a mesh or other fabric (13) can be laid down on the first over-coating film together with polymer suspended cells by either the hydrophilic membrane method or the casting method. The mesh or fabric can optionally be treated in such a manner as to improve bonding to the polymer. Examples of such treatment are covalent modification of the fabric material to allow coupling of polyaminoacids or other polycations and amination. Wettability of the fabric can also be improved by chemical modification, corona treatment or pre-wetting with dilute alginate or other polymer solution and drying.

An alternative means of improving mechanical strength of the sheet is to incorporate a cuff of mesh or other fabric by similar means to provide an outer annulus of support, which can be used for suturing the sheet onto a vascularized surface (see Fig. 22). The integral cuff can be placed so as to be distant from the cells within the core and can be imposed in such a way as to ensure its total encapsulation within the coating polymer layers to improve biocompatibility. Alternatively, the cuff

can be imposed in such a way as to allow presentation to cells of the implant recipient to encourage engraftment of the sheet without stimulation of cellular deposition within the immediate environment of the contained cells.

In another alternative, the polymer solution of the coat or core can contain microfibrillar collagen, fibrin or other microfibrillar material, which will enhance mechanical strength.

Treatment of fully cross-linked thin polymer layers or a fully cross-linked cell-containing polymer core with multi-functional reagents such as poly-lysine, poly-asparagine or other poly-cationic polymers provides another means of improving mechanical strength, while simultaneously providing another means of controlling permeability. The presence of unreacted reactive moieties of polymer-bound multi-functional reagent provides a means for bonding between the so treated cross-linked polymer layer and the layer of soluble polymer (coat or core, interchangeably). Following application of coats to core, immersion in cross-linker stabilizes the entire sheet. In the case of multi-functional reagent treatment of the core (as opposed to the coating layers) an advantage is afforded by the fact that the multi-functional reagent so applied will be buried beneath the over-lying polymer layer and thus not exposed to the recipient, which may react to said multi-functional reagent.

A number of other optional materials may be co-entrapped with cells in the sheet. For example, immobilized enzymes may be desirable for modification of sloughed or secreted cellular substances or immobilized hemoglobin or fluorocarbon fluid may be included to improve oxygen and CO_2 solubility and transport through the gel matrix. It may be desirable to add materials which will be released slowly over time, such as agents to minimize inflammation in response to the initial trauma of surgical implantation.

It may also be desirable to co-encapsulate different cell types, where such cells are capable of interacting in some desirable fashion. For example, inclusion of a "feeder" cell may improve viability or survival of a particular cell. Another example is the use of Sertoli cells to induce tolerance (U.S. Patent No. 5,849,285, Selawry, Helena P).

Inclusion of materials which promote engraftment and/or neovascularization may also be desirable. For example, hyaluronic acid may be included in the coat. For example, a cell line that secretes known vascularization factors may be included in the core and/or coat or materials that encourage vascular growth in adjacent tissues e.g., VEGF.

Insolublized or entrapped enzymes which catalyze desirable conversion of naturally present substances in the recipient may also be entrapped alone within a sheet.

In order to minimize abrasion of the device and to maintain it in intimate contact with a well vascularized site within the recipient, the implant may be sutured to the omentum or trapped beneath an omental flap sutured to the surface of a vascularized organ (Fig. 21). A support cuff can optionally be sutured into place exterior to the space between the omental patch and the organ. A sheet can be sutured onto any vascularized site including the surface of the liver or other organs or subcutaneously. Optionally, the device may be held in place during engraftment by fibrin glues, preferably autologous plasma concentrates. Treatment with serum, plasma, platelet releasate or other compositions which promote healing may offer the additional advantage of diminishing foreign body reaction and scar formation.

Figs. 17-21 show various views of thin sheets. Fig. 17 shows a sheet with a red cell containing core. Fig. 18 shows an electron micrograph of the surface of an islet sheet; the islets inside appear as convex areas of the surface. Fig. 19 shows an electron micrograph of a sheet reinforced with nonwoven fabric mesh, fractured; the flat surface (11); the edge of the broken upper half of the sheet (12); the mesh (13) and the lower half of the sheet (14). Fig. 20 shows a sheet retrieved after over 2 months in a dog and Fig. 21 shows an islet sheet made by the method of Example 8 being attached to the dog's omentum.

Fig. 22 shows blood sugars following implant of a canine Islet Sheet into a pancreatectomized diabetic dog of Example 8. Fig. 23 shows intravenous glucose tolerance tests (IVGTT) of the same dog 30 days and 60 days after the implant.

Example 1

Preparation of guluronate-rich alginate

Guluronate-rich alginate was purified by a modification of the method of Dorian, et al. (U.S. Patent No. 5,643,594; http://www.isletmedical.com/meth0202.htm (March 31, 1999)). Briefly, 1 gram Protan MVG alginate was dissolved in 1 liter 0.5 mM EDTA, 10 mM HEPES, pH 7.0. The solution was filtered to 0.45 microns to remove particulates then mixed with 4 grams bleached, activated charcoal for 30 minutes to adsorb organic contaminants (bleaching of charcoal comprised stirring 30 minutes in 100 mL 0.1 M sodium perchlorate then washing by centrifugation 2x times with 100 mL H2O, 4 times with 100 mL EtOH, 4 times with 100 mL H2O). The charcoal adsorbed alginate was filtered sequentially to 0.22 microns then 0.1 microns. To the filtered solution was added 10.2 mL 10% $MgCl_2 \cdot 2H_2O$. While stirring, 3.8 mL of 34% $CaCl_2 \cdot 2H_2O$ was added to precipitate larger, guluronate-rich chains. After 30 minutes of stirring, the precipitate was pelleted and the supernatant was discarded.

The pellet was dissolved in 150 mL 0.1 M EDTA disodium salt, 10 mM HEPES, pH 7.0. After dilution to 1 liter with water, the solution was filtered three times by concentrating ten fold in a 17 kD hollow fiber cartridge. The retentate was diluted to 250 mL with water and NaCl was added to a final concentration of 100 mM. While stirring vigorously, an equal volume of anhydrous ethanol was added slowly. The precipitated alginate was then pelleted by centrifugation, dissolved in 250 mL 100 mM NaCl, reprecipitated by addition of an equal volume alcohol as above and pelleted. After 2 more identical washes with 50% alcohol, the final pellet was resuspended in 40 mL 50 mM NaCl and combined with 160 mL ethanol, pelleted and washed 3 times by centrifugation with 200 mL ethanol to remove water, salts and residual organic contaminants. After the last wash, the pellet was pressed to remove excess alcohol, teased and fluffed with forceps and dried overnight in vacuo at 60 degrees.

Example 2

Preparation of mannuronate-rich alginate

Mannuronate-rich alginate was purified by a modification of the method of Dorian, et al. (U.S. Patent No. 5,429,821; http://www.isletmedical.com/meth0102.htm (March 31, 1999)). One gram Kelco HV alginate was dissolved in 1 liter 0.5 mM EDTA, 10 mM HEPES, pH 7.0. The solution was filtered to 0.45 microns then mixed with 4 grams bleached, activated charcoal for 30 minutes (charcoal bleached as in example 1). The charcoal adsorbed alginate was filtered sequentially to 0.22 microns then 0.1 microns. The filtered solution was then filtered with a hollow-fiber cartridge and alcohol washed as in example 1. After the last wash, the pellet was pressed to remove excess alcohol, teased and fluffed with forceps and dried overnight in vacuo at 60 degrees.

Example 3

Preparation of Sheets directly on smooth glass surface

Purified canine islets were sedimented by gravity from serum free medium and washed twice by gentle centrifugation with a wash buffer comprised of 0.9% NaCl, 3 mM glucose, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0. The washed islets were resuspended in a 3% solution of purified mannuronate-rich alginate (example 2) in 0.9% NaCl, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0.

A solution of soluble alginate salt (3% hi-M in normal saline/10 mM HEPES, pH 7.0) is spread as a thin layer on a perfectly smooth surface, such as optical glass (Borofloat Window, Edmund Scientific cat# K45-686. A layer was formed by dragging the alginate across the smooth surface with a straightedge (precision stainless steel straightedge, McMaster-Carr cat# 2215A2) while controlling the thickness by means of guide shims fabricated from 75 micron polycarbonate film. A reinforcing fabric (Hollytex, Ahlstrom cat# 3251) was placed on top of the alginate layer. An additional 187.5 um shim was stacked atop the 75 um shim. The suspension of islets in alginate solution was spread with a straightedge across the surface of the layer.

A coating layer of alginate (2% hi-M in normal saline/10 mM HEPES, pH 7.0) was spread with a straightedge over the top of the islet-containing layer. The entire laminate was submerged in a solution of cross-linking ions (1.7% $CaCl_2 \cdot 2H_2O$, 10 mM HEPES. pH 7.0) to cross-link the alginate.

Example 4

Preparation of non-reinforced sheets by hydrophilic membrane method

Partially cross-linked layers of alginate (6 cm wide and 20 cm long) were prepared by spreading to a depth of 125 microns a 5% solution of 0.45 micron sterile filtered purified mannuronate-rich alginate (example 2) in 0.9% NaCl, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0 onto a 0.2 micron nitrocellulose filter membrane (Micro Filtration Systems) which had been pre-wetted with 4.63% sucrose, 3.2% calcium gluconate, 10 mM HEPES, pH 7.0 and blotted before alginate application. Backing the filter membrane at its underside was a hinged pair of plates of optical glass positioned relative to the membrane in such a way that the membrane and spread alginate layer spanned the hinged junction of the 2 plates. Control of the thickness of the spread alginate layer was achieved by using a straightedge guided by shim rails to sweep the alginate onto the wet membrane surface. The shim rails were then removed.

Two hundred microliters of 0.45 micron sterile filtered 5% solution of purified guluronate-rich alginate (example 1) in 0.9% NaCl, 3 mM glucose, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0 were dispensed from a syringe onto the layer in the approximate center of one of the hinged plates. The hinged plates were then pivoted toward each other so that the alginate layer on the membrane filter folded into juxtaposition, squeezing the droplet of guluronate-rich alginate into a disk entrapped within a sandwich of under- and over-lying alginate. The thickness of the sandwich was determined by spacers which held the two plates at a defined distance from each other. The plates were then hinged apart, leaving the membrane/alginate layer/guluronate-rich core/alginate layer/membrane sandwich, overlaid by an optional thin polycarbonate film lying on one of the 2 plates. The plastic film provided a dry interface to break the surface tension which otherwise binds the plates together, and was peeled off and the overcoated sheet trapped

within the folded membrane was transferred into a dish of 1.7% $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, pH 7.0 to complete cross-linking of the alginate. One fifth volume of 115 mM $BaCl_2$ was added to the fixative solution and the sheet was incubated for an additional 5 minutes. Finally, the sheet was rinsed with 5 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, pH 7.0 and transferred to serum free DMEM culture medium with added 3 mM $CaCl_2 \cdot 2H_2O$ and 10 mM HEPES, pH 7.0 in normal saline for maintenance during shipment to the surgical facility.

Example 5

Encapsulation of islets in paper-reinforced sheets by hydrophilic membrane method

Purified canine islets were sedimented by gravity from serum free medium and washed twice by gentle centrifugation with a wash buffer comprised of 0.9% NaCl, 3 mM glucose, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0. The washed islets were resuspended in a 5% solution of purified guluronate-rich alginate (example 1) in 0.9% NaCl, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0.

Partially cross-linked layers of alginate (6 cm wide and 20 cm long) were prepared by spreading to a depth of 125 microns a 5% solution of purified mannuronate-rich alginate (example 2) in 0.9% NaCl, 0.5 mM sodium citrate, 10 mM HEPES, pH 7.0 onto a 0.2 micron nitrocellulose filter membrane (Micro Filtration Systems) which had been pre-wetted with 4.63% sucrose, 3.2% calcium gluconate, 10 mM HEPES, pH 7.0 and blotted before alginate application. Backing the filter membrane at its underside was a hinged pair of plates of optical glass positioned relative to the membrane in such a way that the membrane and spread alginate layer spanned the hinged junction of the 2 plates. A 25 micron thick sheet of polycarbonate film had been interposed between the filter membrane and one of the optical glass plates prior to spreading of the alginate layer. Control of the thickness of the spread alginate layer was

achieved by using a straightedge guided by shim rails to sweep the alginate onto the wet membrane surface.

A 5 cm disk of 25 micron thick polyester non-woven scrim (Ahlstrom) was placed onto the layer in the approximate center of one of the hinged plates. Two hundred microliters of the islet suspension in alginate were dispensed from a syringe onto the polyester disk. The hinged plates were then pivoted toward each other so that the alginate layer on the membrane filter folded into juxtaposition, flattening the droplet of suspended islets into a disk entrapped within a sandwich of overlying alginate. The thickness of the sandwich was determined by spacers which held the two plates at a defined distance from each other. The plates were then hinged apart, leaving the membrane/alginate layer/islet suspension/alginate layer/membrane sandwich, overlaid by the thin polycarbonate film lying on one of the 2 plates. The plastic film was peeled off and the overcoated sheet trapped within the folded membrane was transferred into a dish of 1.7% $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, pH 7.0 to complete cross-linking of the alginate. One fifth volume of 115 mM $BaCl_2$ was added to the fixative solution and the sheet was incubated for an additional 5 minutes. Finally, the sheet was rinsed with 5 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, pH 7.0 and transferred to serum free DMEM with added 3 mM $CaCl_2 \cdot 2H_2O$ and 10 mM HEPES, pH 7.0 for maintenance during shipment to the surgical facility.

Example 6

Preparation of VEGF-containing sheets

Sheets containing Vascular-Endothelial Growth Factor (VEGF) complexed to sucralfate, an α-D-glucopyranoside, ß-D-fructofuranosyl-, octakis-(hydrogen sulfate), aluminum complex, were constructed by the following method. The dry sucralfate/VEGF complex when added to the alginate suspension at 400 mg/ml formed a thick paste which could not be swept easily over the pure alginate layer. The paste was applied to a

polyester scrim in a smear of uniform 500 μm thickness. A 75 μm thick layer of liquid 3% alginate was made by sweeping the alginate suspension with a straightedge and shim. The paste and embedded scrim were carefully lifted with filter forceps and gently laid atop the liquid alginate layer. A second layer of liquid 2% alginate was swept over the paste/scrim with a straightedge guided by a 750 μm thick shim. This sandwich was gelled by overlaying the liquid alginate with a solution of 1.7% $CaCl_2 \cdot 2H_2O$. After 5 minutes the gel sandwich was lifted from the glass substrate and transferred to a solution of 7mM $CaCl_2$ in normal saline until implanted in rats.

Example 7

Preparation of Agarose Sheets

Agarose 2% (SIGMA Agarose Type I-B Low EEO) was suspended in normal saline/10 mM HEPES, pH 7.0 and heated in a water bath to 60°C to dissolve it. The agarose was cooled to 45°C (at which temperature it remains liquid) in a water bath and divided into two aliquots. Dextran beads (Sephadex G-50) were suspended in one solution 10% v/v to serve as mock islets and maintained at 45°C.

The solution of agarose without mock islets was spread as a thin layer on optical glass (Borofloat Window, Edmund Scientific cat# K45-686). A layer was formed by dragging the hot agarose across the smooth surface with a straightedge (precision stainless steel straightedge, McMaster-Carr cat# 2215A2) while controlling the thickness by means of guide shims fabricated from 75 micron polycarbonate film. The layer was allowed to cool for a few seconds and solidify. An additional 187.5 um shim was stacked atop the 75 um shim. The 45°C suspension of dextran beads in agarose solution was spread with a straightedge across the surface of the layer and allowed to cool briefly. The two shims were removed and replaced with a single polycarbonate shim 300 microns thick.

A coating layer of agarose was spread with a straightedge over the top of the bead-containing layer using the shims as guides. The entire laminate was allowed to cool to room temperature then submerged in a solution of saline/10 mM HEPES, pH 7.0.

Example 8

Biocompatibility test of non-reinforced sheets in mice, rats and dog

Sheets prepared as described in Example 5 were implanted intraperitoneally in mice, subcutaneously in rats, and on the omentum of a dog (Fig. 21). The mice were sacrificed after 3 weeks and the implants removed for histological analysis. The rats were sacrificed after 3 weeks and the implants removed for histological analysis. The dog was sacrificed after 13 days and the implants removed for histological analysis. The explanted sheets were physically intact and minimal foreign body reaction was observed.

Example 9

Implantation of islet containing sheets into a pancreatectomized dog

A total of 200 microliters of islets were obtained from 2 mongrel dogs by standard collagenase treatment and Ficoll density gradient purification. Islets were divided into 6 aliquots and encapsulated in polyester reinforced sheets prepared as described above in Example 3. The sheets were implanted into a pancreatectomized beagle on the dog's omentum (Fig. 21).

Management of the dog's blood sugars required a single injection of 2 units insulin on day 9 (Fig. 22). Fasting blood sugars remained in the normal range for 10 weeks.

Eighty-four days after implantation the sheets and adjacent omentum were removed. Seven days later the dog had become diabetic with fasting blood sugar over 200 mg/ml. The sheet was wet mounted and frozen for histology. Examination of the sheets before fixation showed viable islet cells (by Trypan Blue exclusion) containing insulin (Fig. 20) as assessed by dithizone stain.

At 30 and 60 days an intravenous glucose tolerance test was performed according to the standard protocol. Briefly, the dog is injected with 7 ml 50% glucose, and blood sugars are measured at the times shown in the table below.

Dog	Beagle	Total Pancreatectomy/Allograft	
Body weight	7 kg		
IV 50% Dextrose	7 mL	30 days	60 days
Sample #	Sample time (min)	Glucose (Beckman) (mg/dL)	Glucose (Beckman) (mg/dL)
1	-5	87	86
2	0	94	79
3	1	299	279
4	5	258	236
5	10	219	211
6	20	187	169
7	30	177	144
8	45	145	117
9	60	136	97
10	90	95	84
11	120	93	79

The results of these tests are shown in Fig. 23. The IVGTT was closer to normal at 60 days compared to 30 days showing an increase in sheet function.

WHAT IS CLAIMED IS:

1. A method of making a physiologically active and biocompatible cellular implant for implantation into a host body, said method comprising the steps of:

(a) forming first and second layers of first and second polymers, respectively, each layer having a first substantially uncross-linked polymer solution surface and an opposing second cross-linked surface,

(b) forming a sandwich of a cell suspension layer comprising a cell suspension layer of physiologically active cells in a substantially uncross-linked third polymer solution between said first and second layers at their respective first surfaces so that their respective interfaces are in liquid form, and

(c) cross-linking said first and second polymer solutions in a direction from the second surface of one or both of said first or second layers to the cell suspension layer until the first and second polymer of said first and second layers, respectively, and said cell suspension layer are cross-linked at their respective interfaces, thereby forming a cellular implant.

2. The method of claim 1 in which the interface cross-linking of said first, second and third polymer is performed by gelling.

3. The method of claim 2 in which said first, second and third polymer solutions comprise polysaccharide.

4. The method of claim 3 in which said first, second and third polymer solutions comprise alginates.

5. The method of claim 1 in which said cross-linking is performed by diffusing cross-linking agent from said second surface of one or both of said first or second layers to said cell suspension layer.

6. The method of claim 5 in which said cross-linking agent comprises multivalent cations.

7. The method of claim 5 in which said cross-linking agent is selected from the group consisting of calcium, barium, strontium or mixtures thereof.

8. The method of claim 5 in which said cross-linking agent comprises a calcium salt.

9. The method of claim 6 in which said multivalent cations are in said salt form and the counterion for the multivalent cation in the salt is selected from the group consisting of gluconate, lactate, chloride or mixtures thereof.

10. The method of claim 2 in which the temperature of step (c) is different from the temperature of step (b) and said gelling occurs in response to the change of temperature from step (b) to step (c).

11. The method of claim 10 in which said first, second and third polymer solutions comprise agarose.

12. The method of claim 1 in which said first, second and third polymer solutions comprise photoactivatable cross-linking groups, said cross-linking comprising photoactivation of said photoactivatable cross-linking groups.

13. The method of claim 12 in which said first, second and third polymer solutions comprise polyethylene glycol derivatized with photolabile cross-linking groups.

14. The method of claim 1 in which said cells comprise secretory cells.

15. The method of claim 14 in which said secretory cells comprise islets of Langerhans.

16. The method of claim 1 in which said active cells in said third polymer solution are first encapsulated in microcapsules or hollow fibers.

17. The method of claim 1 in which in step (b) said first and second layers are deposited directly or indirectly on first and second plates, respectively, said method further comprising pressing said plates toward each other to form said cell suspension layer.

18. The method of claim 17 in which during pressing said first and second plates are precisely spaced apart from each other by at least one spacer of predetermined thickness during step (b).

19. The method of claim 17 in which said first and second layers are separated from said first and second plates by first and second flexible membranes, respectively, to form a membrane-jacketed implant comprising a multi-layer of first membrane - first layer - cell suspension layer - second - membrane.

20. The method of claim 19 in which said first and second membranes include first and second smooth polymer layers, respectively, facing and in contact with said first and second layers.

21. The method of claim 19 in which the membrane is selected from the group consisting of agarose, polyacrylamide, alginate, polyethylene glycol or mixtures thereof.

22. The method of claim 19 further comprising the step of removing said membrane-jacketed implant as a unit from said first and second plates.

23. The method of claim 22 further comprising contacting said plate-removed membrane-jacketed implant with a solution of said cross-linking agent.

24. The method of claim 22 in which said contacting is performed by immersing said plate-removed membrane-jacketed implant in a solution of said cross-linking agent.

25. The method of claim 1 in which said first, second and third polymer solutions are identical.

26. A method of making a physiologically active and biocompatible cellular implant for implanting into a host body, said method comprising the steps of

(a) forming a first layer of a first substantially uncross-linked polymer solution of predetermined thickness by substantially uniformly spreading said first polymer solution on a solid support surface, said first layer having an exposed surface,

(b) forming a cell suspension second layer of predetermined thickness on said first layer exposed surface, said cell suspension comprising physiologically active cells in a second substantially uncross-linked second polymer solution, by substantially uniformly spreading said cell suspension over said first layer exposed surface, and

(c) forming a third layer of a third substantially uncross-linked polymer solution on said cell suspension second layer by substantially uniformly spreading said third polymer solution on said cell suspension second layer.

27. The method of claim 26 in which said third polymer has a high viscosity than said second polymer layer and said second polymer has a higher viscosity than said first polymer.

28. The method of claim 26 in which forming said first, second and third layers is performed by depositing said first, second and third polymer solutions successively with spacers of first, second and third increasing thicknesses sequentially placed to extend from said solid support surface; and sequentially sliding a substantially flat edge across said first spacer in step (a), across said second spacer in step (b) and said third spacer in step (c).

29. The method of claim 27 further comprising diffusing cross-linking agent through said first, second and third layers to form a sandwich-type thin cellular implant and removing said implant from said support surface.

30. A method of treating a disease in which a cellular implant made by the method of claims 1 or 26 is implanted into a vascularized site in a host.

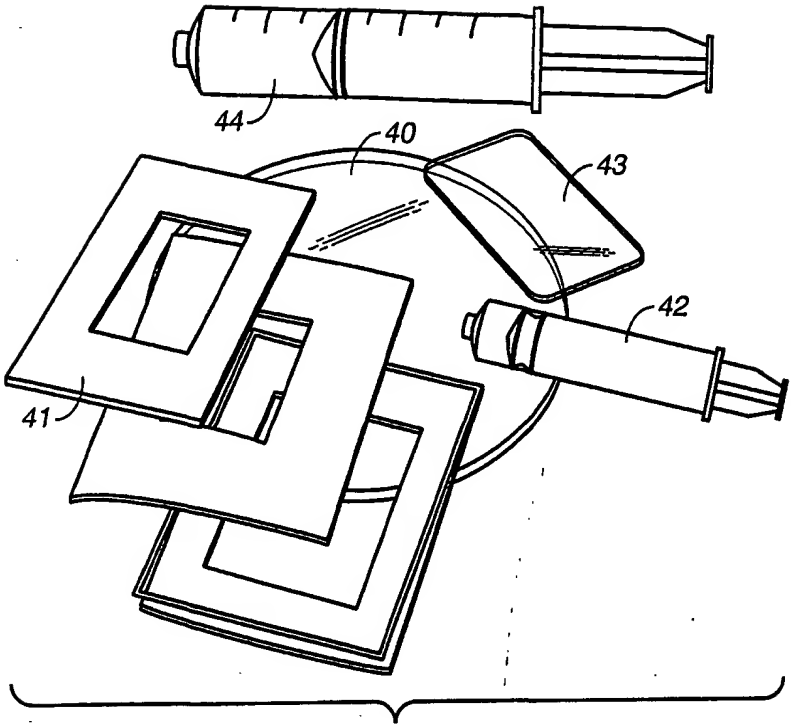

FIG._1

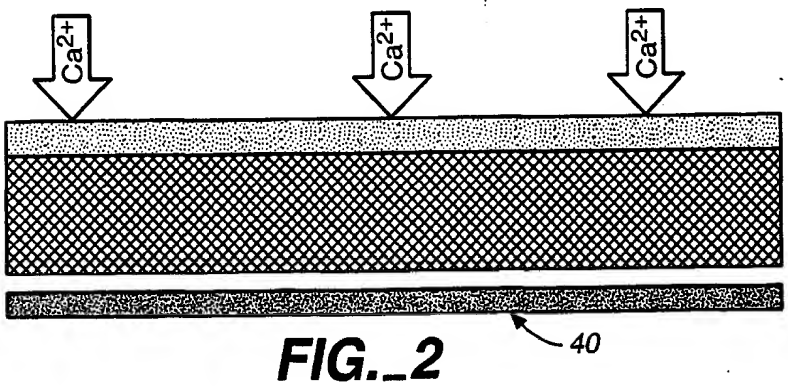

FIG._2

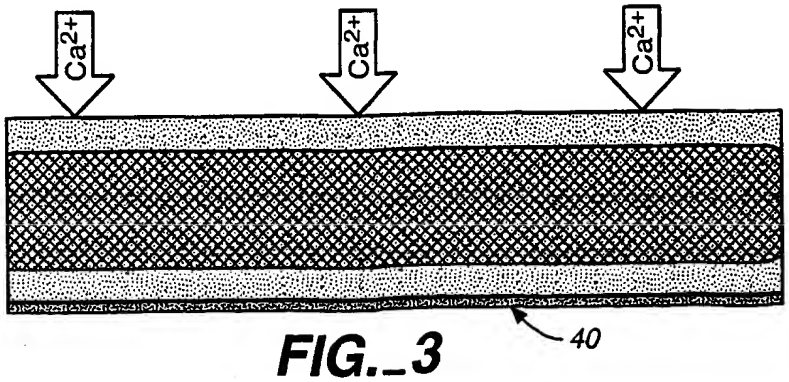

FIG._3

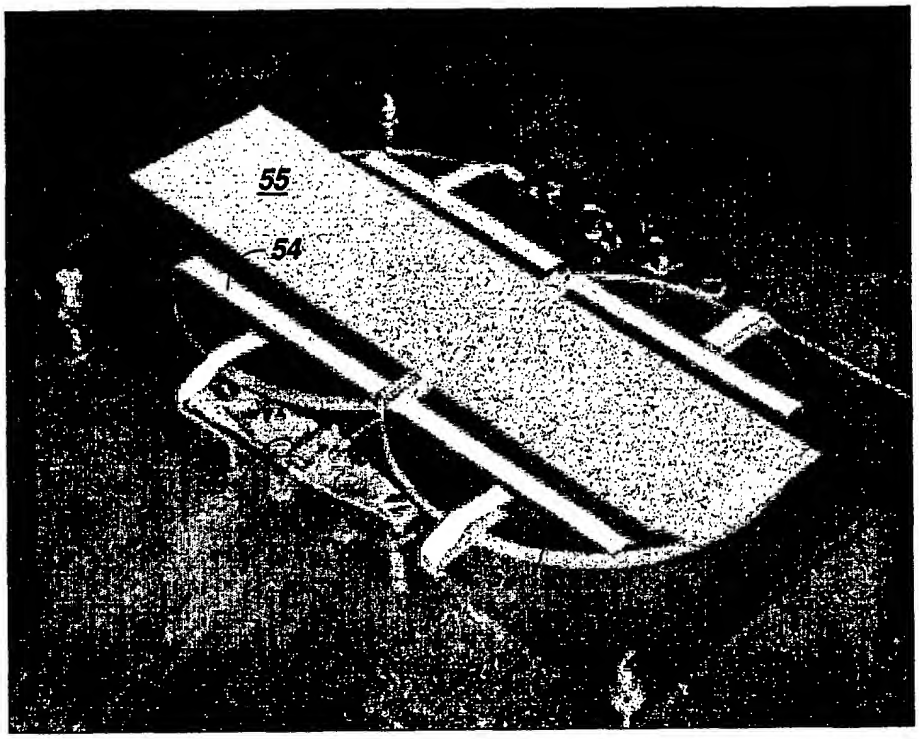

FIG._4

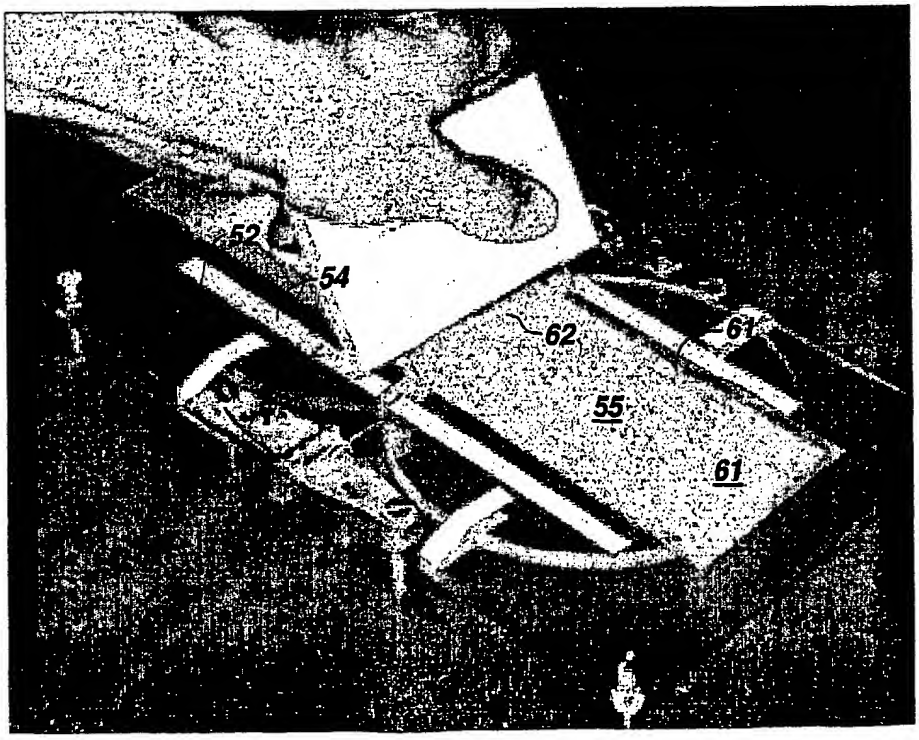

FIG._5

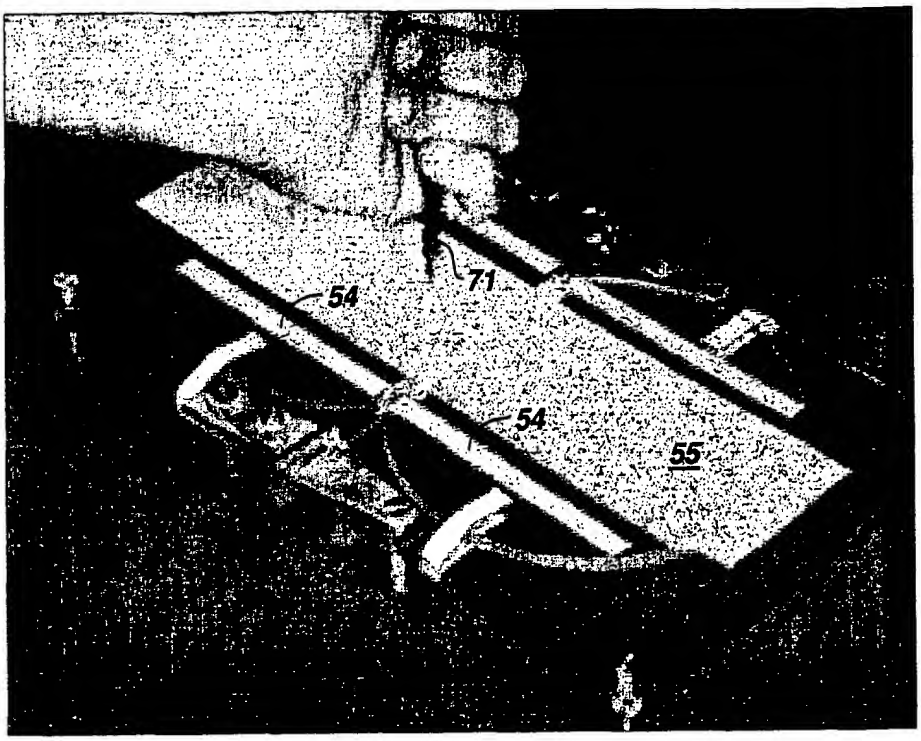

FIG._6

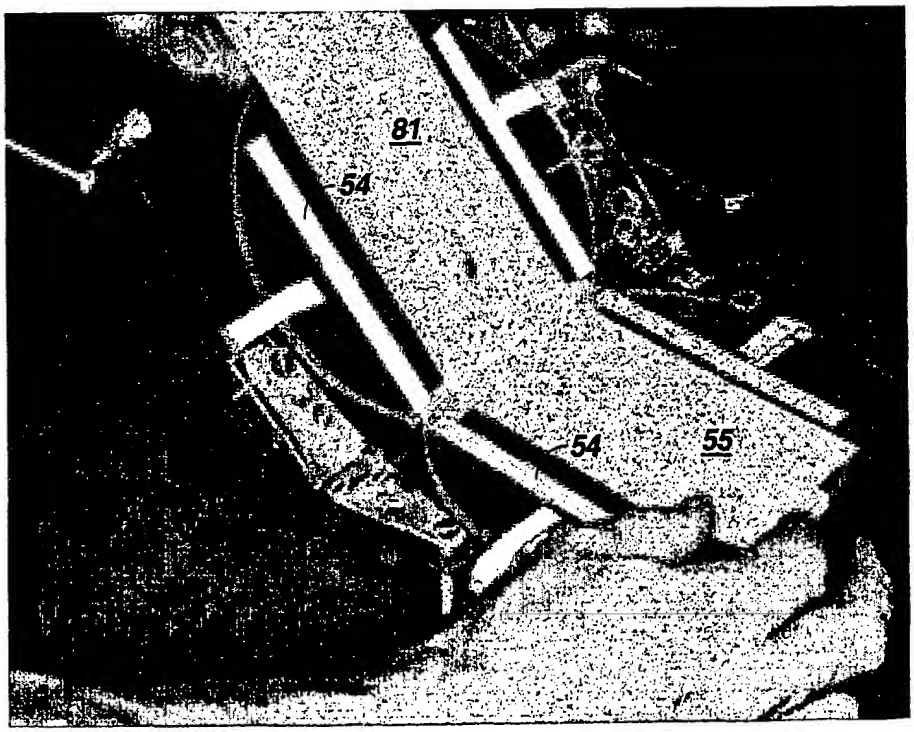

FIG._7

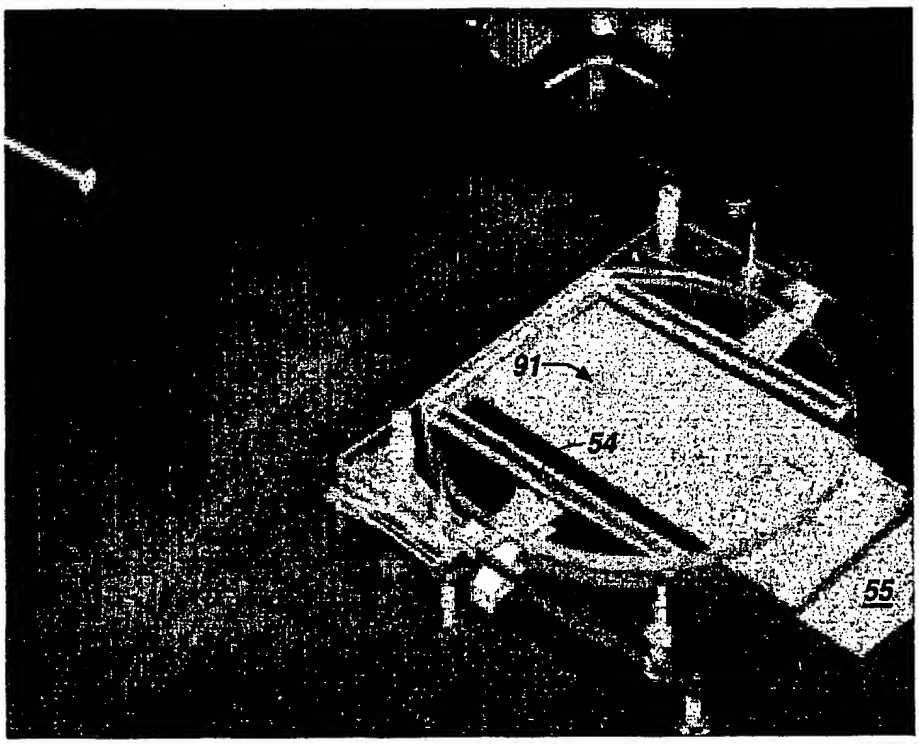

FIG._8

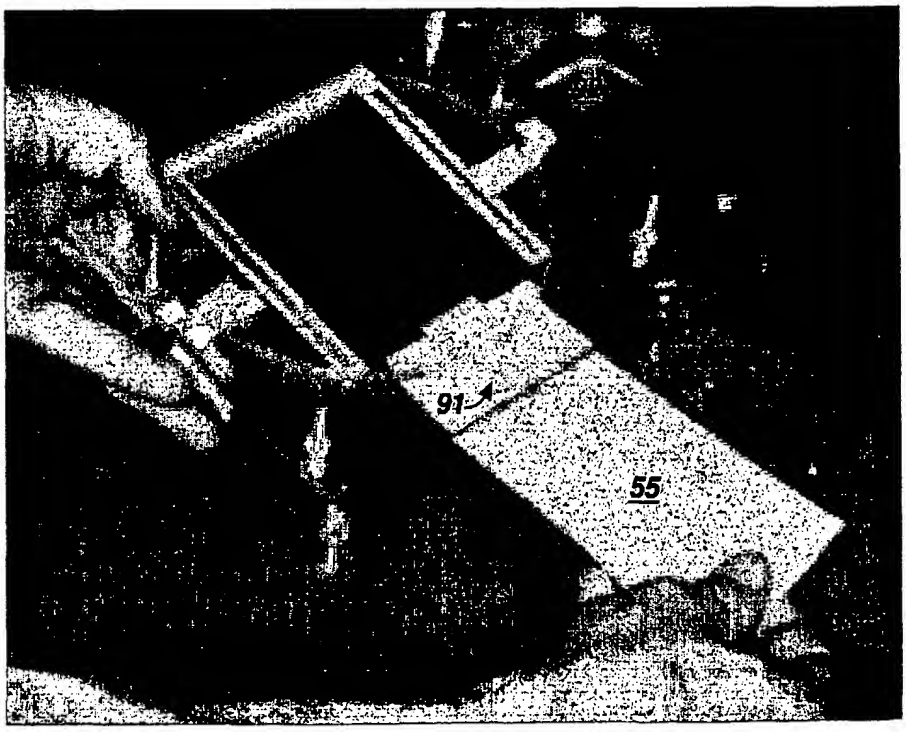

FIG._9

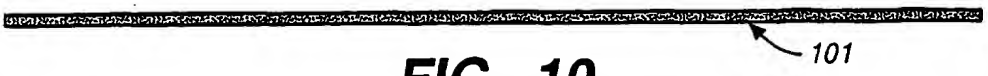

FIG._10

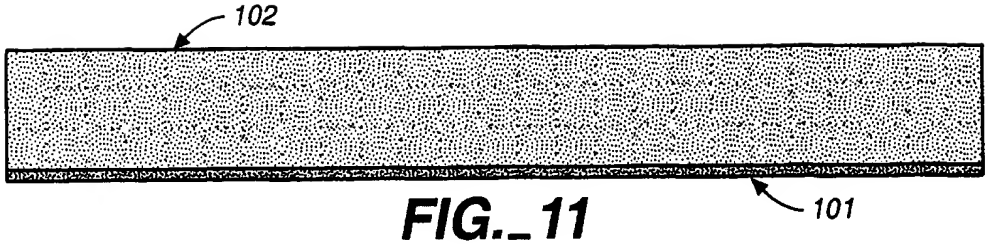

FIG._11

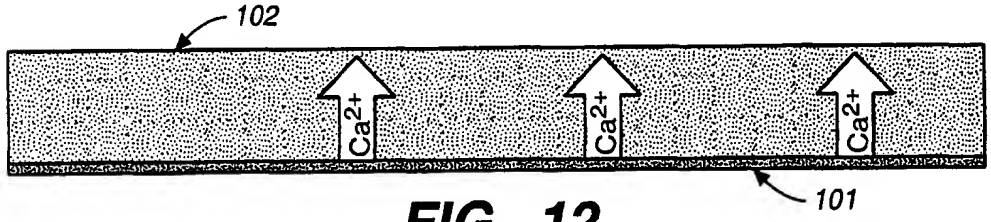

FIG._12

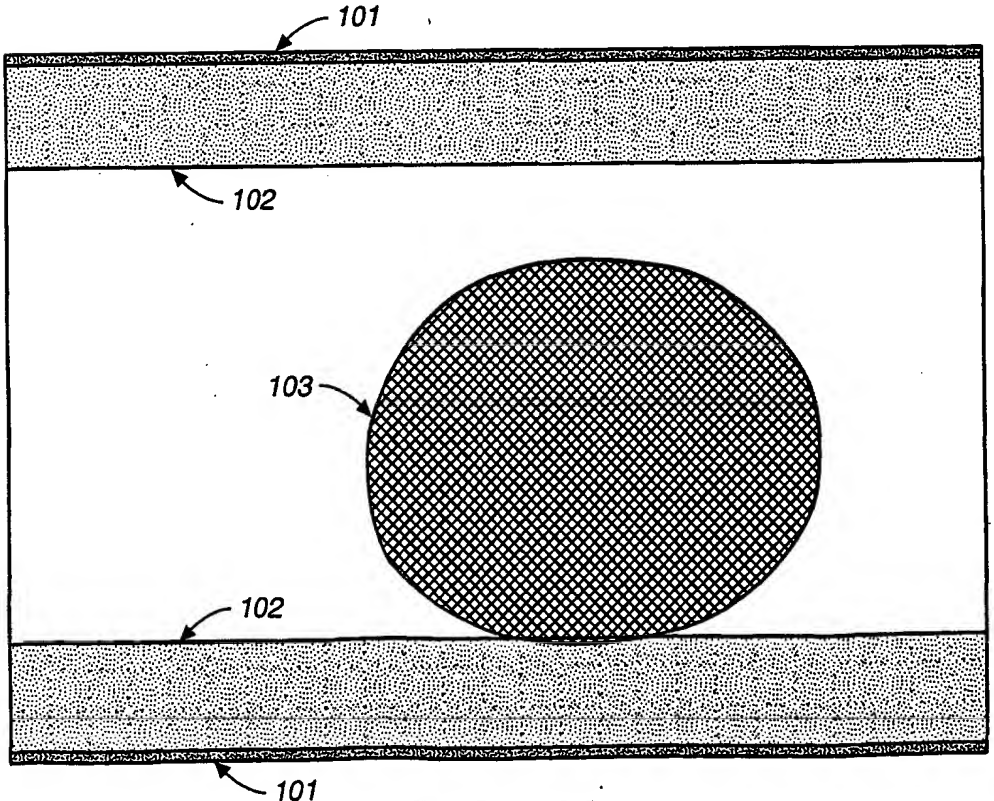

FIG._13

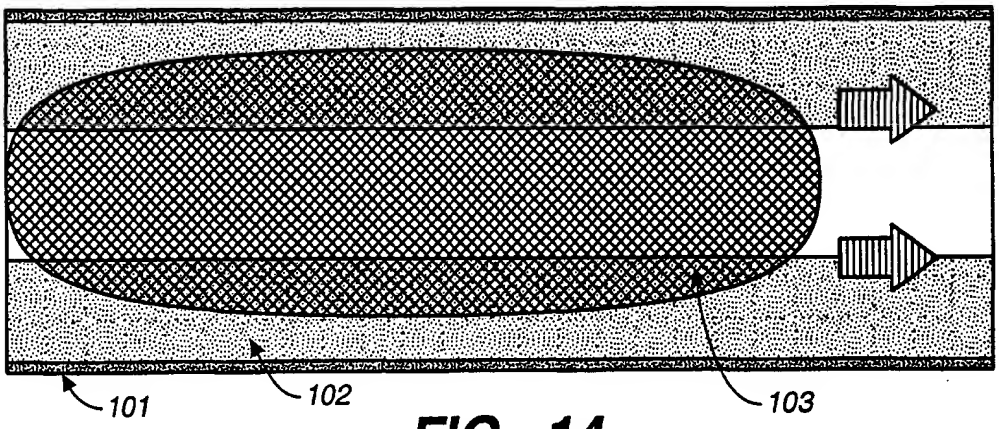

FIG._14

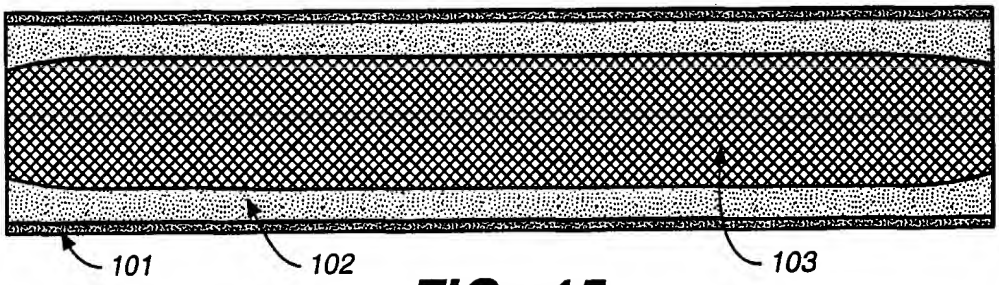

FIG._15

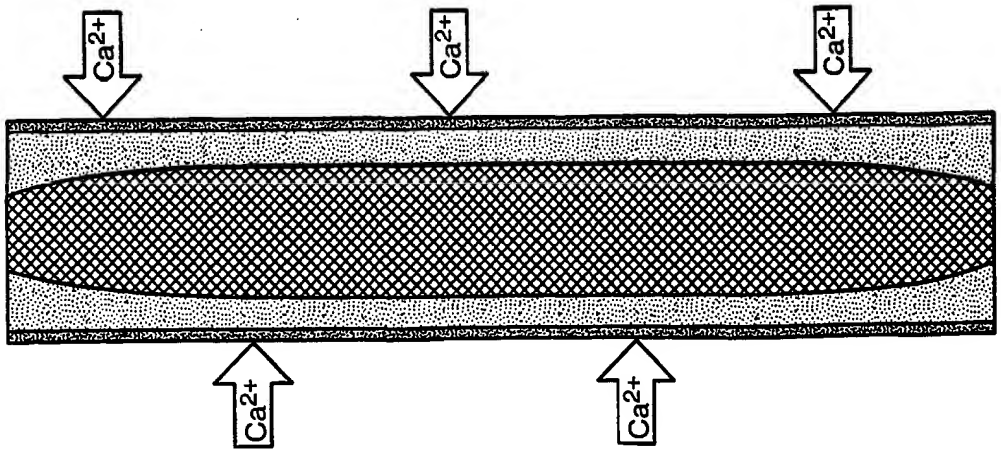

FIG._16

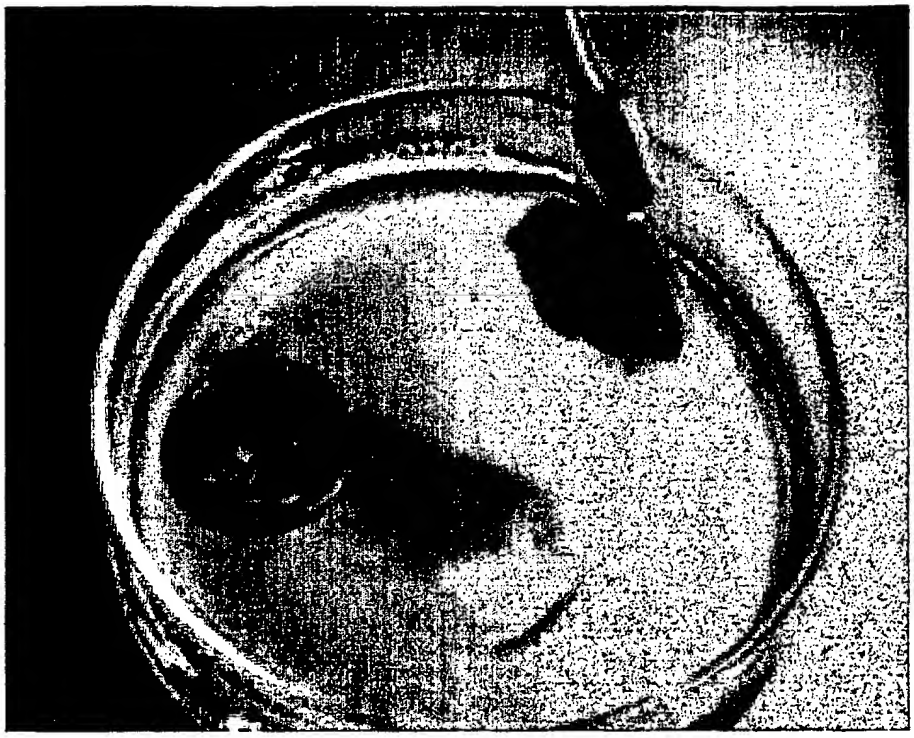

FIG._17

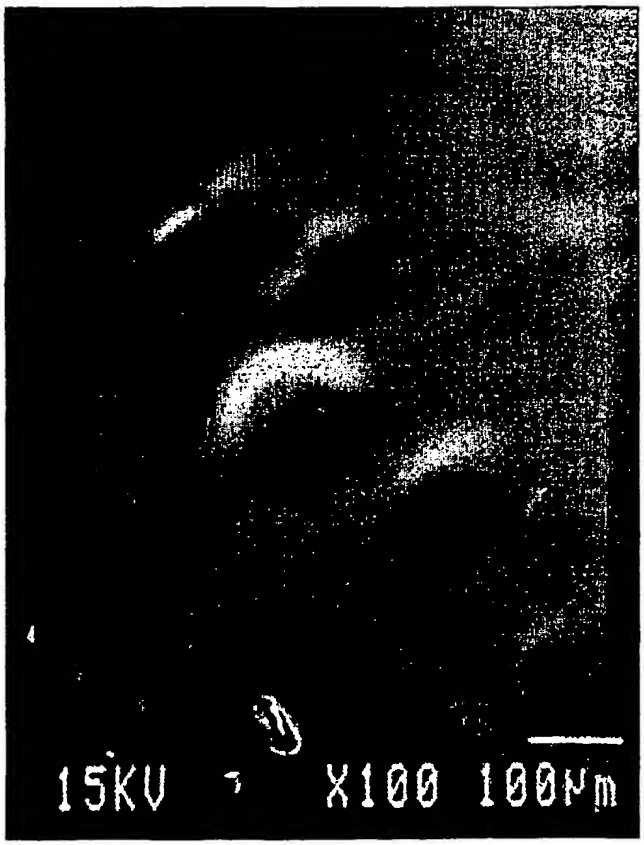

FIG._18

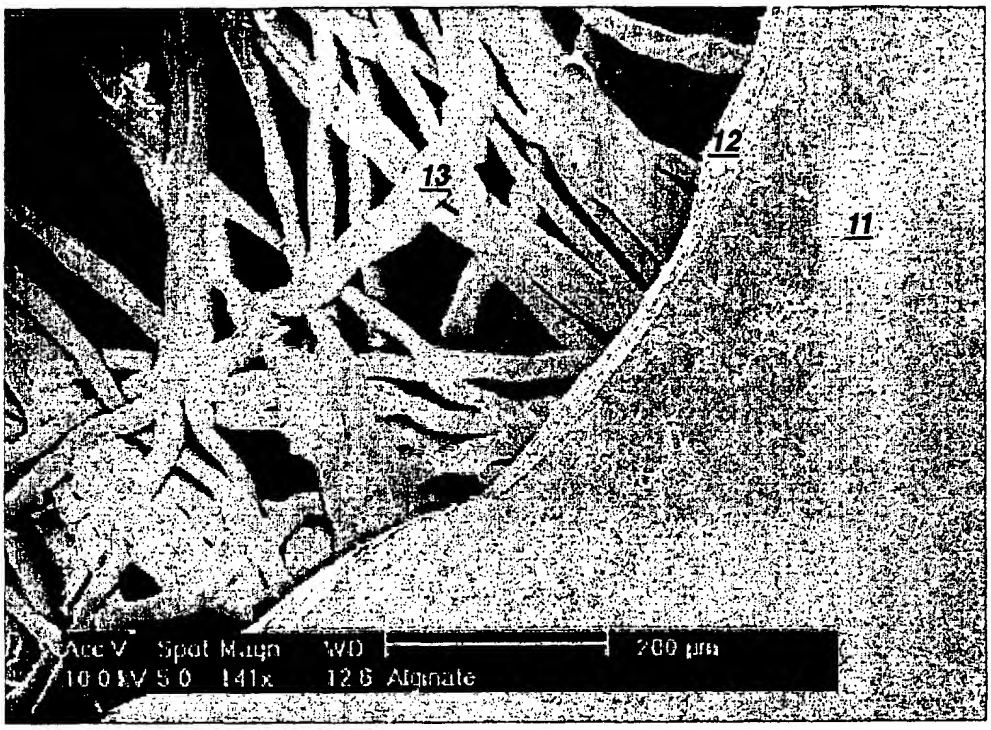

FIG._19

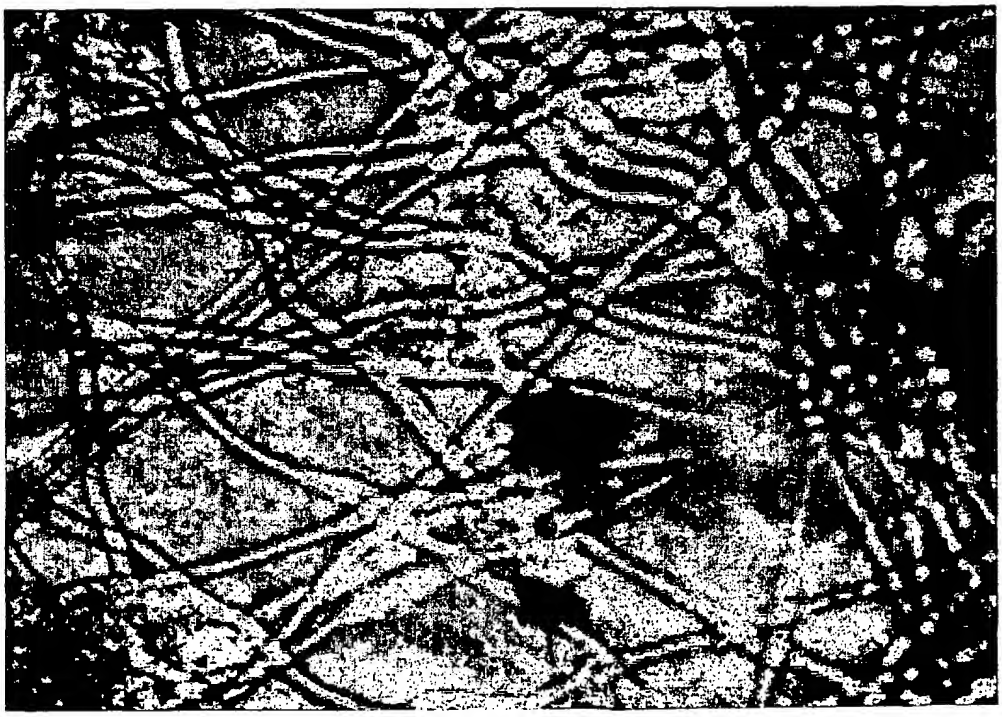

FIG._20

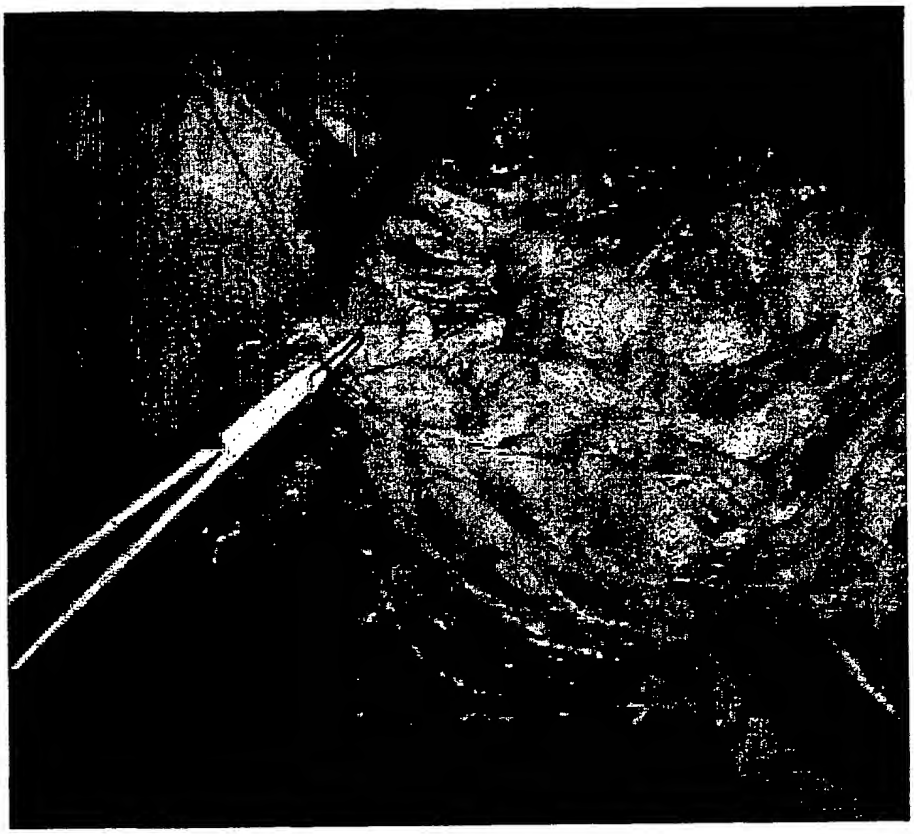

FIG._21

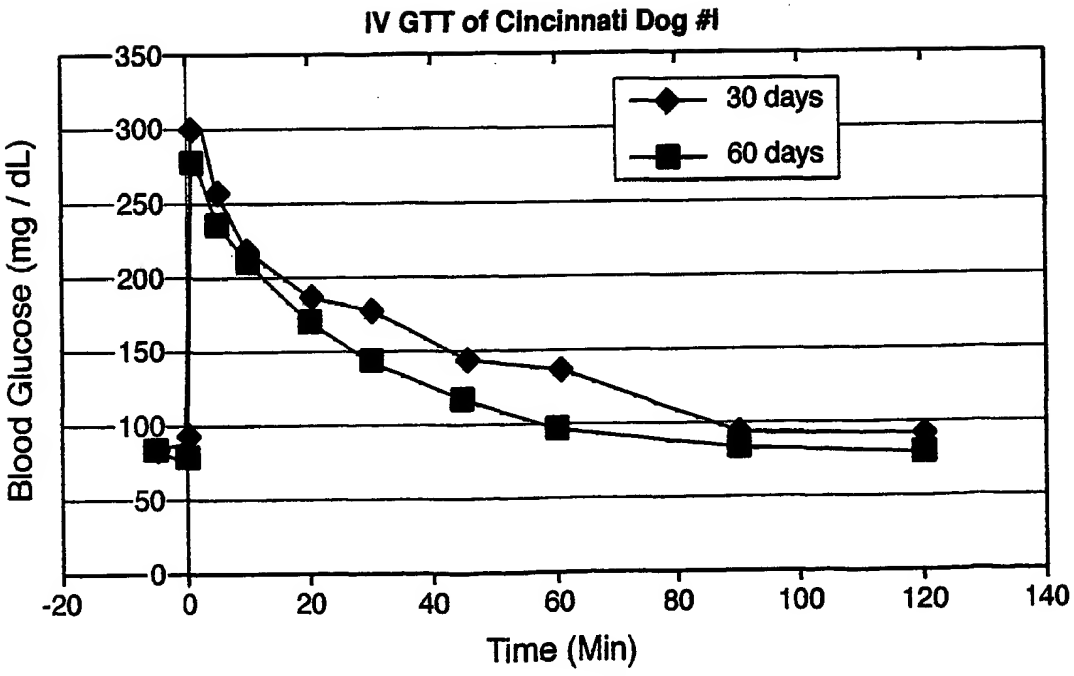
IV GTT of Cincinnati Dog #I
30 days
60 days
Blood Glucose (mg / dL)
350
300
250
200
150
100
50
0
-20
0
20
40
60
80
100
120
140
Time (Min)

FIG._23

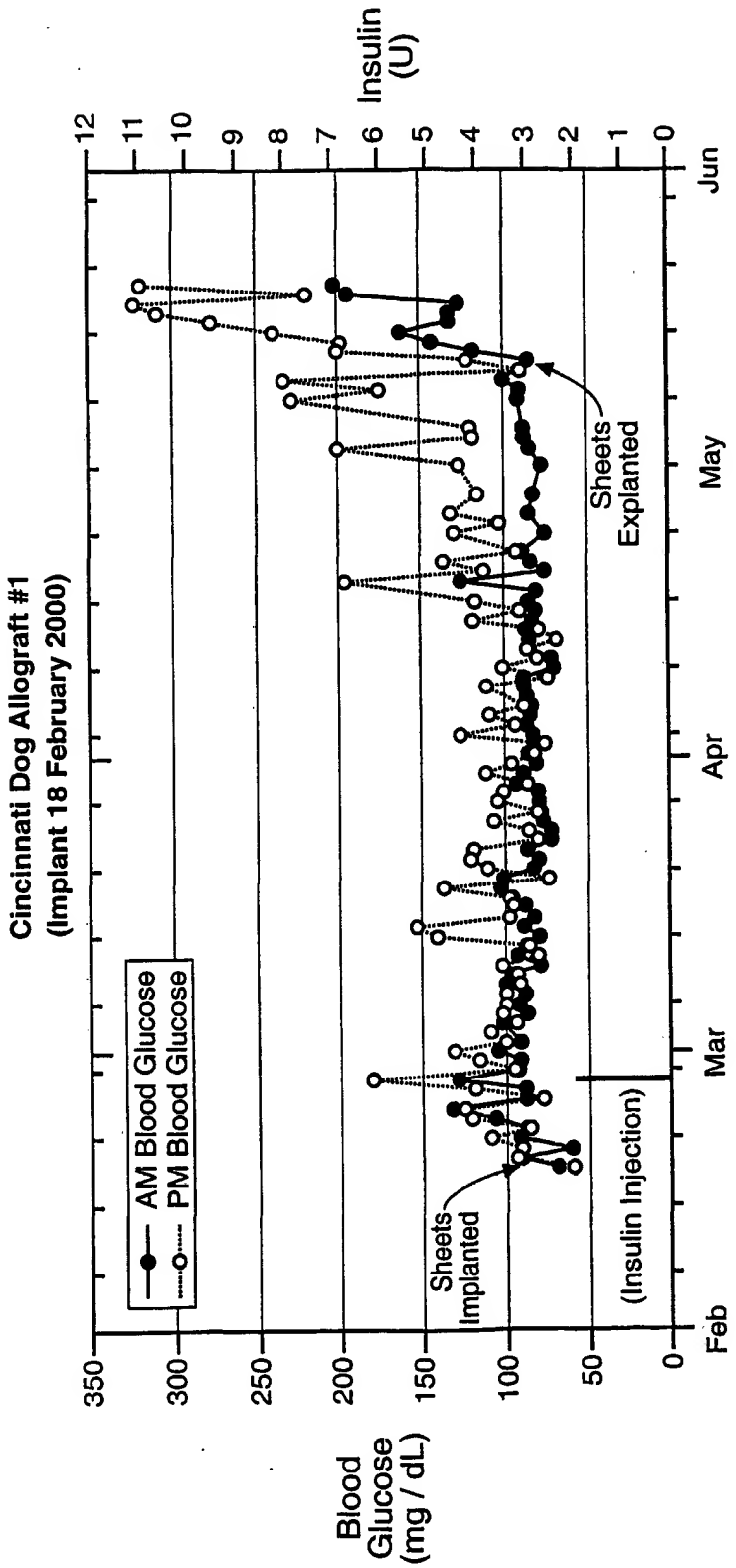
Cincinnati Dog Allograft #1
(Implant 18 February 2000)
Blood Glucose (mg / dL)
Insulin (U)
AM Blood Glucose
PM Blood Glucose
Sheets Implanted
Sheets Explanted
(Insulin Injection)
Feb
Mar
Apr
May
Jun
350
300
250
200
150
100
50
0
12
11
10
9
8
7
6
5
4
3
2
1
0

FIG._22